Saskatchewan Agriculture, Food and Rural Revitalization

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AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

19990248

DEVELOPMENT OF PORCINE ADENOVIRUS-3 BASED VACCINES

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FINAL REPORT

SASKATCHEWAN AGRICULTURE DEVELOPMENT FUND (PROJECT #19990248)

"DEVELOPMENT OF PORCINE ADENOVIRUS-3 BASED VACCINES"

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SUMMARY

Vaccination against infectious diseases is considered to be one of the major advances in modern medicine when one considers the reduction in human and animal suffering. Sub-unit vaccines often do not induce breadth of immunity required (e.g. cell mediated immunity etc.), and they require strong adjuvants, of which few are licensed. Furthermore, producing sub unit vaccines for use in veterinary medicine, using modern biotechnology approaches is dictated by economics. Thus, novel approaches are required to produce animal vaccines to reduce animal suffering and productivity. One feasible way of achieving this is to develop safe and effective and economically viable live-vectored vaccines. The main objective of this grant was to develop porcine adenovirus -3 (PAV-3) based live viral vector. The PAV-3 infects pigs but often does not produce disease. Earlier, we characterized PAV-3 at the molecular level by determining the complete genome sequence and transcriptional map.

Now, we have identified different essential and non essential regions / sites of PAV-3 genome, which can be deleted and used for insertion of foreign genes. In addition, we have constructed a replication competent PAV-3 vector which can accommodate additional 5 kb foreign DNA. We have also demonstrated the feasibility of constructing PAV-3s expressing reporter genes or vaccine antigens of porcine respiratory and reproductive syndrome virus (PRRS). In order to demonstrate the feasibility of this approach, we performed the challenge experiments in a natural host. Our preliminary results suggest that immunization of pigs with recombinant PAV-3 expressing PRRS antigens did not protect animals in protection / challenge experiments. However, further experiments need to be done before full potential of this approach (PAV-3 vector) can be realized.

TECHNICAL REPORT

a) Background

The use of vaccines is an effective method of stimulating the naïve animal to develop protective immunity against the disease causing organism. Since many disease causing organisms enter at mucosal surfaces (respiratory and gastrointestinal tract), immunity is required at the mucosal site to block the initial infection and thus reduce the chances of development of disease. As compared to sub-unit vaccines, live vaccines are best inducers of mucosal immunity when administered orally or intranasally. In addition, production of live vaccines is cost effective. However, use of live vaccines produced by conventional means ensures that the live organism is always present in the animals which many times can mutate back to virulent form (in vivo recombination) and cause fatal disease. Thus, new approaches have to be developed for the safe and cost effective production of viral vaccine antigen. One way to achieve this is to develop live vectored vaccines. Such live vectors can be engineered to carry genes for other pathogens thus making it possible to immunize animals to produce protective immunity at the mucosal surface to various disease organisms at one time. This will not only improve vaccination, but will also reduce the cost of producing vaccines. We have choosen porcine adenovirus-3 (PAV-3) for the development of such vaccines as it infects porcine respiratory and gastrointestinal tract and induced mild disease[1,2]. By genetically engineering this virus, we can cripple (E3 deletion) it so that it will not cause disease. It can be further crippled by an E1 deletion so that it can infect and produce the proteins but not assemble into the virus particles. Introduction of foreign genes (respiratory / enteric viruses of porcine) into different regions of PAV-3 genome, will not only reduce the

pathogenicity of the virus, but will also make PAV-3 as a safe and effective vehicle for delivering vaccine antigens at the mucosal surfaces. We have choosen porcine respiratory and reproductive disease of pigs as a model since it causes significant economic losses due to pneumonia in young pigs, reproductive failures in sows and an increase in preweaning mortality[3].

b) Project Objectives

The long term goal of this grant is to develop cost effective, safe and efficacious mucosal vaccines[4] against diseases of porcine. The specific aims of this projects are:

1) Constructing mutant PAV-3 deleted in E1, E3 or E4 region.
2) Testing of virulence and immunogenicity of E1,E3 and E4 deleted PAV-3
3) Construction of recombinant PAV-3 expressing vaccine antigens (e.g. ORF5) of PRRS virus.
4) Testing the immunogenicity and protection against challenge in pigs

c) Results and Discussion

Earlier, we sequenced the genome of porcine adenovirus-3 and determined the transcriptional map (Fig. 1a,b). This helped us to locate the regions (e.g. E1, E3 and E4), which can be used for the deletion / insertion of foreign genes. In addition, we have also developed an easy method of constructing recombinant PAV-3. Using homologous recombination machinery of *E.coli*, we constructed a full length PAV-3 genomic clone in

a plasmid (pFPAV200) (Fig. 2a). This plasmid DNA when transfected into porcine cells produced virus, which was identical to wild type PAV-3 (Fig. 2b).

i) Construction of recombinant PAV-3 with

E3 deletion. To delete E3 region, thePAV3 genome between nt 27402 and 28112 (PCR1) and between nt 28709 and 29859 (PCR2) was amplified using PCR. PCR1 was digested gigestedwith BamHI. The PCR2 was digested with BamHI and SpeI. The two gel purified fragments were cloned into SpeI-SmaI sites of plasmid pBluescript by three way ligation (Fig. 3) creating plasmid pPAV300. An 1868 bp SphI-SpeI fragment flanking E3 region was isolated from pPAV300 and recombined with SnaBI digested pFPAV200 (using homologous recombination machinery of *E. coli*.BJ5183) creating plasmid pFPAV300 (Fig. 3). The PacI digested plasmid DNA was trasfected into fetal porcine retina cells. The cytopathic effects were observed in 7 days. The recombinant named PAV300 was plaque purified, amplified and virion DNA was isolated. The restriction enzyme digestion of virion DNA confirmed the identity of the recombinant PAV300 (Fig. 4).

E1A deletion. Since E1 region is necessary for the viral replication, recombinant PAV-3 missing E1 region can only be isolated and propagated in cell lines expressing E1 proteins. Initially, we attempted to isolate a cell line expressing E1 region proteins of PAV-3 without success. To isolate a cell line, which could complement the E1defect of PAV3, we transformed fetal porcine retina cells with E1 region of human adenovirus-5 (HAV5). The transformed cell clones were tested for expression of E1 proteins by

Western blot analysis and for replication of E1deleted HAV5. Based on expression of E1 proteins and complementation of E1 deleted HAV5, we selected a cell line designated as VIDO R1 (Fig. 5). To construct a deletion in E1 region, first we constructed a transfer plasmid pPAV101 by ligating 5.5 kb fragment isolated after digestion of pFBAV200 with NcoI. The pPAV102 was digested with NotI and AseI , blunt end repaired by T4 DNA polymerase and ligated to SrfI linker creating plasmid pPAV102. The plasmid pFPAV201 (Fig. 6) was created by co-traformation of *E. coli* BJ5183 cells and NoI-linearized pPAV102 and the genomic DNA of PAV-3 with a deletion in E3 region. The PacI digested pFPAV201DNA when tranfected into VIDO R 1 cells produced cytopathic effects in 23 days. The recombinant virus named PAV201 was plaque purified, amplified and virion DNA was isolated. The restriction enzyme analysis of virion DNA (Fig. 7) confirmed the identity of the recombinant virus. As recombinant PAV201 grows on VIDO R1 but not on fetal porcine retina cells suggesting that E1 is essential for replication of PAV3 in vitro.

E1B^{small} deletion A 9.225 kb XhoI fragment (containing vector backbone plus left [nt 004159] and right [nt 31053 to 34094] termini of PAV-3 genome) isolated from plasmid pFPAV200 (Reddy et al., 1999a) was religated creating plasmid pPAVXhoIRL. A 633 bp fragment (nt 827 to 1460) isolated by PCR amplification (using oligonucleotides YZ-17 5'-ACA GTA ATG AGG AGG ATA TC-3' and YZ-18 5'-TAG GAC TAG TCC CAC AGA AAA AGA AAA GG-3' as primers and plasmid pPAVXhoIRL as a template) was digested with EcoRV - SpeI and gel purified. A 403 bp fragment (nt 1820 to 2223 of PAV-3 genome) isolated by PCR amplification (using oligonucleotides YZ-19 5'-ATG GAC TAG TCT TCT GGT GCC GCC ACT A -3' and

YZ-20 5'-CCT AAT CTG CTC AAA GCT G-3' as primers and plasmid pPAVXhoIRL DNA as a template) was digested with SpeI - Eco47III and gel purified. A 6.947 kb XhoI - StuI fragment of plasmid pPAVXhoIRL was blunt end repaired with T4 polymerase and religated to create plasmid pYZ9a. The two gel purified DNA fragments were ligated to EcoRV - Eco47III digested plasmid pYZ9a in a three way ligation. The resulting plasmid pYZ21 contains 360 bp deletion (nt 1460-1820) in $E1B^{small}$ region and an engineered SpeI site. Finally, a 5.506 kb HpaI - AspI fragment of pYZ21 was ligated to 3.374 kb HpaI - AspI fragment of pPAVXhoIRL to create plasmid pYZ21a. The recombinant PAV-3 genome containing deletions in the $E1B^{small}$ and the E3 region (pFPAV212) (Fig. 8a) was generated by homologous DNA recombination in *E. coli* BJ5183 between XhoI linearized pYZ21a and the genomic DNA from PAV E3. PacI digested pFPAV212 DNA transfected into VIDO R1 cells produced cytopathic effects after after 7-10 days. The identity of the recombinant virus was confirmed by restriction enzyme analysis of virion DNA. (Fig. 8b).

$\underline{E1B^{large}\text{ insertion.}}$ Plasmid pPAVXhoIRL was digested partially with Eco47III and ligated to SpeI linker (triple phase stop [TPS] codon). Plasmid pYZ9 containing SpeI linker inserted in $E1B^{large}$ ORF was selected. The recombinant PAV-3 genome containing deletion in E3 and insertion in $E1B^{large}$ (pFPAV507) was generated by homologous DNA recombination machinery in *E. coli* BJ5183 between XhoI linearized pYZ9 and the genomic DNA from PAV E3. PacI digested pFPAV507 DNA (Fig. 9) transfected into VIDO R1 cells did not produce cytopathic effects after several attempts. These results suggested that $E1B^{large}$ is essential for replication of PAV3.

E1A-E1B^{small}+E3 deletion: . A 0.591 kb BamHI - AseI fragment was excised from plasmid pYZ20 and ligated to 5.309 bp BamHI - AseI (partial) digested pYZ21 to create plasmid pYZ36. Finally, a 4.813 kb HpaI - AspI fragment excised from plasmid pYZ36 was ligated to 3.373 kb HpaI - AspI fragment of plasmid pPAVXhoIRL to create plasmid pYZ37. The recombinant PAV-3 genome containing deletions in E1A, E1B^{small} and E3 region (pFPAV214) was generated by homologous recombination in *E. coli* BJ5183 between XhoI linearized plasmid pYZ37 and genomic DNA from PAV E3 . The full length plasmid pFPAV214 (Fig. 10a) contained 727 bp (nt 530 -1230) deletion in E1A, 360 bp (nt 1460 - 1820) deletion in E1B^{small} and 597bp (nt 27405 - 28112) deletion in E3. PacI digested pFPAV214 DNA transfected into VIDO R1 cells produced cytopathic effects after 7-10 days. The identity of recombinant virus was confirmed by restriction enzyme analysis of virion DNA (Fig. 10b).

E1b^{large} deleted: Earlier, we suggested that E1b^{large} is essential for PAV-3 replication.In order to isolate a recombinant PAV-3 containing E1b^{large} deletion / insertion, we first constructed a cell line expressing PAV-3 E1B^{large} by transfecting VIDO R1 cells with a plasmid containing PAV-3 E1B^{large} ORF under the control of CMV promoter and hygromycin resistance gene fused to IRES sequence of EMC virus. The hygromycin resistant clones were analyzed by RT-PCR and by Western blot analysis for the expression of PAV-3 E1B^{large} protein. The clone (named VR1BL) expressing high amount of E1B^{large} protein was selected (Fig. 11). Transfection of VR1BL with PacI digested pFPAV507 DNA produced cytopathic effects in two weeks. The identity of the recombinant virus named PAV507 was confirmed by restriction enzyme analysis of virion DNA (12). This confirmed that E1B^{large} is essential for PAV-3 replication in vitro.

E1A+E1B^{small}+E1B^{large} deletion: Finally, we attempted to construct a recombinant PAV-3 containing **complete deletion of E1** (E1A+E1b^{small}+E1B^{large}). Taking advantage of homologous recombination machinery of *E. coli*, we constructed a plasmid pFPAV227 containing full-length PAV-3 genomic DNA with deletion of E1 (nt 524-3274) and E3 deletion (nt 28112-28709). Transfection of VR1BL cells with PacI digested pFPAV227 DNA produced cytopathic effects in two weeks. The identity of the virus (named PAV227) was confirmed by restriction enzyme analysis of virion DNA and Western blot analysis of E1 proteins in virus infected cells (Fig. 13). These results suggested that VR1BL cell line can complement the growth of recombinant PAV227 containing complete deletion of E1 region.

E4 insertion. Initially we determined if region between E4 and the right inverted terminal repeat can be used for the insertion of a foreign gene, we constructed a transfer vector pPAV400 by ligating 7 kb XhoI fragment obtained after digestion of pFPAV200 with XhoI. The cholamphenicol acetyl (CAT) gene under the control of SV40 immediate-early promoter and polyadenylation signal was inserted into HpaI site of pPAV400 creating plasmid pPAV400.CAT. The XhoI-PacI fragment containing CAT gene from plasmid pPAV400.CAT was recombined with the HpaI digested pFPAV200 by homologous recombination in *E. coli* BJ5183 creating plasmid pFPAV400-CAT (Fig. 14a). The PacI digested pFPAV400-CAT DNA was transfected into swine testicular (ST) cells. The cytopathic effects were observed in 7 days. The recombinant virus named PAV3-CAT was plaque purified, amplified and virion DNA was isolated. The restriction enzyme digestion of the virion DNA confirmed the identity of the recombinant virus (Fig. 14b).

E4 deletion. In order to determine if E4 is essential for virus replication, we constructed E4 transfer vectors in which we deleted the individual ORFs (ORF1 to ORF7). Using homologous recombination machinery of *E. coli*, we constructed PAV-3 full length DNA clones containing complete deletion of each of the PAV-3 E4 ORFs (ORF1 to ORF7). The PacI digested individual DNA clones when transfected in VIDO R1 cells produced viral plaques except the full length PAV-3 DNA clone containing complete deletion of E4 ORF3 (Fig. 15). Repeated transfections of this E4 ORF3 deleted PAV-3 DNA clone in VIDO R1 cells did not produce viral plaques. The identity of other recombinant PAV-3s was confirmed by restriction enzyme analysis of virion DNA (Fig. 16). Using similar approach, we next isolated a recombinant PAV-3 named PAV438 containing deletions in E4 ORF1 to ORF2 & ORF4 to ORF 7. This confirmed that except ORF3, none of the E4 ORFs are essential for replication of PAV-3.

To increase the insertion capacity of our PAV-3 vector to express genes like TGEV S glycoprotein, we have now successfully constructed a replication competent recombinant PAV-3 named PAV700 containing deletions in E3 (nt 28112-28709) and E4 (nt 30302-32666 & nt 33044-33636) region (Fig. 17). These deletions have no effect on the replication of this recombinant PAV700 in vitro as it grows to titers similar to E3 deleted PAV-3. Thus, now we have a vector which can be used to insert upto 5.0 kb foreign DNA. We have now constructed a recombinant named PAV701 expressing a reporter gene in E4 region (Fig. 18). Preliminary characterization of this recombinant suggests that we can use this region (E4) for the expression of vaccine antigens.

ii) Construction of recombinant PAV-3 expressing

Porcine respiratory and reproductive syndrome virus (PRRS) ORF 3. In addition to ORF5, we also synthesized ORF3 gene by using oligonucleotides and Klenow extension reaction. This DNA fragment was digested with BamHI – HindIII and cloned into BamHI – HindIII digested plasmid pSL301 to create plasmid pSLOrf3. A 780 bp BamHI – HindIII fragment of pSLOrf3 was blunt end repaired and ligated to SnaBI digested plasmid pPAV300 (E3 transfer vector) creating plasmid pPAVOrf3. Using homologous recombination machinery of *E. coli*, we recombined this plasmid DNA with SnaBI digested pFPAV300 DNA to create plasmid pFPAV310. This plasmid DNA was digested with PacI and transfected into VIDO R1 cells. The cytopathic effects appeared in two weeks. The identity of the recombinant virus (PAV310) was confirmed by restriction enzyme analysis of viral DNA (Fig. 19).

PRRS ORF5. Using oligonucleotides and Klenow extension reaction, we first synthesized gene encoding PRRS ORF5. This DNA fragment was digested with BamHI – HindIII and cloned it in BamHI – HindIII digested plasmid pSL301 to create plasmid pSLOrf5. A 605 BamHI – HindIII fragment of pSLORF5 was blunt end repaired and ligated to SnaBI digested plasmid pPAV300 (E3 transfer vector) creating plasmid pPAVORF5. Using homologous recombination machinery of *E. coli*, we recombined this plasmid DNA with SnaBI digested pFPAV300. DNA to create plasmid pFPAV312. This plasmid DNA was digested with PacI and transfected into VIDO R1 cells. The cytopathic effects appeared in three weeks. The identity of the recombinant virus (PAV312) was confirmed by restriction enzyme analysis of viral DNA (Fig. 20).

iii) Test the safety and immunogenicity of recombinant viruses

E3 deleted/ E4 insertion. Earlier, it has been shown that deletion of E3 region increases the pathogenicity of human adenovirus-5. Initially, we tried to develop cotton rat as an animal model for these studies. Since, it appeared that cotton rats are not susceptible to PAV3 infection, we proceeded to do these experiments in pigs. Different groups of 3-4 week old piglets were inoculated subcutaneously or oronasally with 10^8 pfu of wild-type, E3 deleted or E4 inserted PAV3s. The animals were observed for development of any clinical signs including temperature and virus replication. Our results suggest that deletion of E3 or insertion of E4 region did not modify the virulence in piglets. Deletion of E3 or insertion in E4 region did not affect the replication of PAV3. Moreover, equivalent levels of antibodies were induced in animals vaccinated by wild type or recombinant PAV3s (as measured by ELISA and virus neutralization assays). These results suggested that recombinant PAV3 deleted in E3, E1a or E4 (insertion) does not affect the virulence of PAV3 and are safe for using as vaccine antigen delivery vehicles in pigs

E1 (complete) deleted PAV-3. Before E1 (complete) deleted PAV3s can be used as vectors, it is important to determine the role of these regions in the pathogenicity of virus in vivo. Different groups of 3-4 week old piglets were inoculated subcutaneously or oro-nasally with 10^8 pfu of wild-type or E1 (complete) deleted PAV3s. The animals were observed for development of any clinical signs including temperature and virus replication. As expected, E1 (complete) deleted virus did not replicate in piglets. Moreover, equivalent levels of antibodies were induced in animals vaccinated by wild

type or recombinant PAV3s (as measured by ELISA and virus neutralization assays). These results suggested that recombinant PAV3 deleted in E1 (complete) does not affect the virulence of PAV3 and are safe for using as vaccine antigen delivery vehicles in pigs.

Test the safety and immunogenicity of recombinant viruses.

Since we could not develop small animal model for testing PAV-3s, we tested the safety and immunogenicity of recombinant PAV-3 expressing PRRS proteins in pigs. Five to six week old crossbred pigs sero negative for PRRS were randomly allocated in different groups. All pigs had low anti-PAV-3 titers in the serum. The pigs were vaccinated twice, 25 days apart with recombinant PAV310, PAV312 or PAV300 either oro-nasally or subcutaneous ($5X10^7$ IU / ml). At day 40, all pigs were challenged intranasally with 10^6 $TCID_{50}$ / ml of PRRS virus (ATCC VR-2332). Serum samples were collected at different times and analyzed for PRRS specific antibodies using HerdCheck PRRS Virus antibody test kit 2XR (IDEXX Laboratories Inc.) and virus neutralization tests.

Very low level of PRRS specific IgG antibodies were detectable before challenge in animals vaccinated oro-nasally. However, after 13 days post challenge, PRRS specific IgG titers were higher in animals immunized with PAV310 or PAV312 compared to control (PAV300) group (Fig. 21). After 21 days post challenge, the PRRS IgG titers of animals immunized with PAV310 or PAV312 were same as control (PAV300) group (Fig. 21). Similarly 13 days post challenge, PRRS specific IgG titers were higher in animals immunized subcutaneously with PAV310 or PAV312 than control (PAV300) group (Fig. 21). Interestingly, PRRS specific IgG titers remained

higher in animals immunized subcutaneously with PAV310 or PAV312 compared to control (PAV300) group (Fig. 21).

At 13 days post challenge, few pigs vaccinated oro-nasally developed PRRS virus neutralization antibodies. At 21 days post challenge, pigs immunized oro-nasally with PAV310 developed higher PRRS virus neutralization response compare to control (PAV300) group (Fig. 22). However, pigs vaccinated subcutaneously developed weak PRRS virus neutralizing antibody response even after 21 days post challenge. Only one pig from each group (PAV310 or PAV312) showed high level of PRRS virus neutralizing antibodies (Fig. 22).

After challenge, pigs developed mild fever. There was no difference in rectal temperatures of animals vaccinated oro-nasally or subcutaneously (Fig. 23). In addition, lungs showed changes consistent with interstitial pneumonia. However, there was no evidence of proliferation of type 2 pneumocytes or necrosis (alveolar or bronchiolar).

f) Conclusions

One of the major impediments to effective vaccines is delivery. We have choosen to develop PAV-3 as a live viral vector for delivery of vaccine antigens. Initially, we developed a simple and efficient way of constructing recombinant PAV-3 (using homologous recombination machinery of *E. coli).* Next, we have identified different regions of the PAV-3 genome (E1, E3, E4), which can be deleted and thus used for the insertion of vaccine antigen genes. This has increased the insertion capacity of replication competent PAV-3 to 5 kb. We have also demonstrated that E1, E3 or E4

deleted PAV-3 viruses are avirulent and immunogenic in animals. In addition, we have demonstrated that properly processed vaccine antigens can be expressed by recombinant PAV-3 vectors.

In order to demonstrate the feasibility of using PAV-3 as a live viral vector, we also performed the challenge experiments in a natural host. Our results suggest that immunization of pigs with recombinant PAV-3 expressing PRRS glycoproteins did not protect the animals from PRRS challenge. However, more studies (e.g. increasing expression level of recombinant protein, evaluating the effect of site of insertion, dose of recombinant virus vector, use of synthetic gene) need to be done to determine the full potential of this approach for delivering vaccines in porcine.

g) References

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BENEFIT TO PRODUCERS

Significant economic losses (mortality, loss of productivity, treatment costs etc.) in the porcine industry are caused by infection. Developing effective preventive measures such as vaccination could prevent these losses. The results of this study suggest that recombinant porcine adenovirus-3 expressing vaccine antigen(s) can be developed and used as a live viral vector for the delivery of vaccine antigens to the mucosal surfaces of porcine. This unique approach of producing new generation of economic, safe and efficacious vaccines should help in greater economic benefits. The oro-nasal route of immunization will prevent not only the meat quality damage (due to intramuscular injections) but will also help to immunize piglets at birth (circumventing the problem of passive immunity).

PERSONNEL

Dr. Suresh Tikoo	Senior Research Scientist
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Dr. P. Wilson	Research Scientist
Dr. Yan Zhou	Research Scientist
Dr. X. Li	PDF
Ms. Caron Pyne	Technician

PROJECT DEVELOPED MATERIALS

a. Zhou, Y., and S. K. Tikoo (2001). Analysis of early region 1 of porcine adenovirus type 3. Virology 291:68-76.

b. Li, X., L. A. Babiuk and S. K. Tikoo.(2003). Functional analysis of E4 region encoded proteins in the replication of porcine adenovirus type 3.(submitted for publication)

c. Li, X. and S. K. Tikoo. (2003). Construction and characterization of E3-E4 deleted recplication-competent porcine adenovirus type 3. 22nd Annual meeting of American Society of Virology. Davis, USA (July 12 – 16)

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GENOMIC ORGANIZATION OF PORCINE ADENOVIRUS TYPE 3 (PAV3)

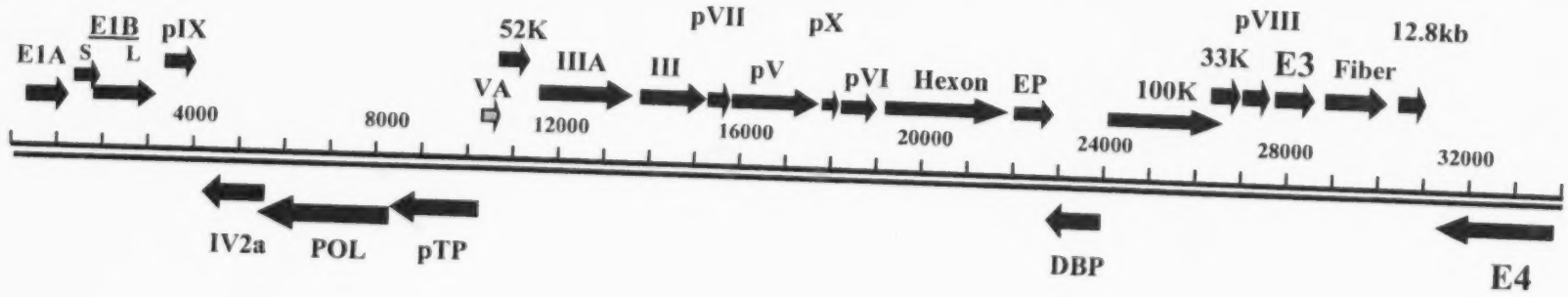


Figure 1a

TRANSCRIPTION MAP OF PORCINE ADENOVIRUS - 3

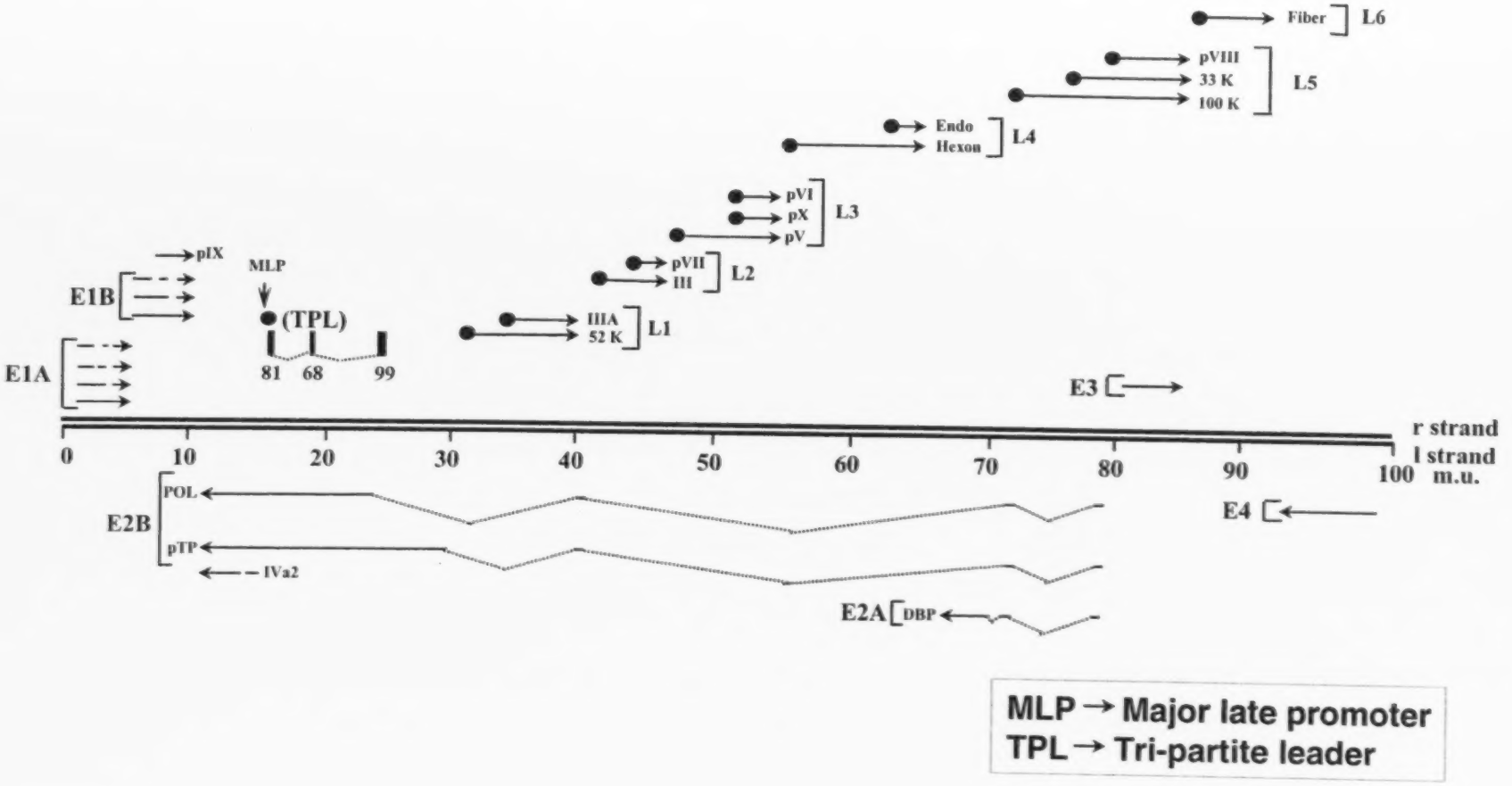


Figure 1b

CONSTRUCTION OF A FULL LENGTH PAV-3 GENOME IN *E. coli*

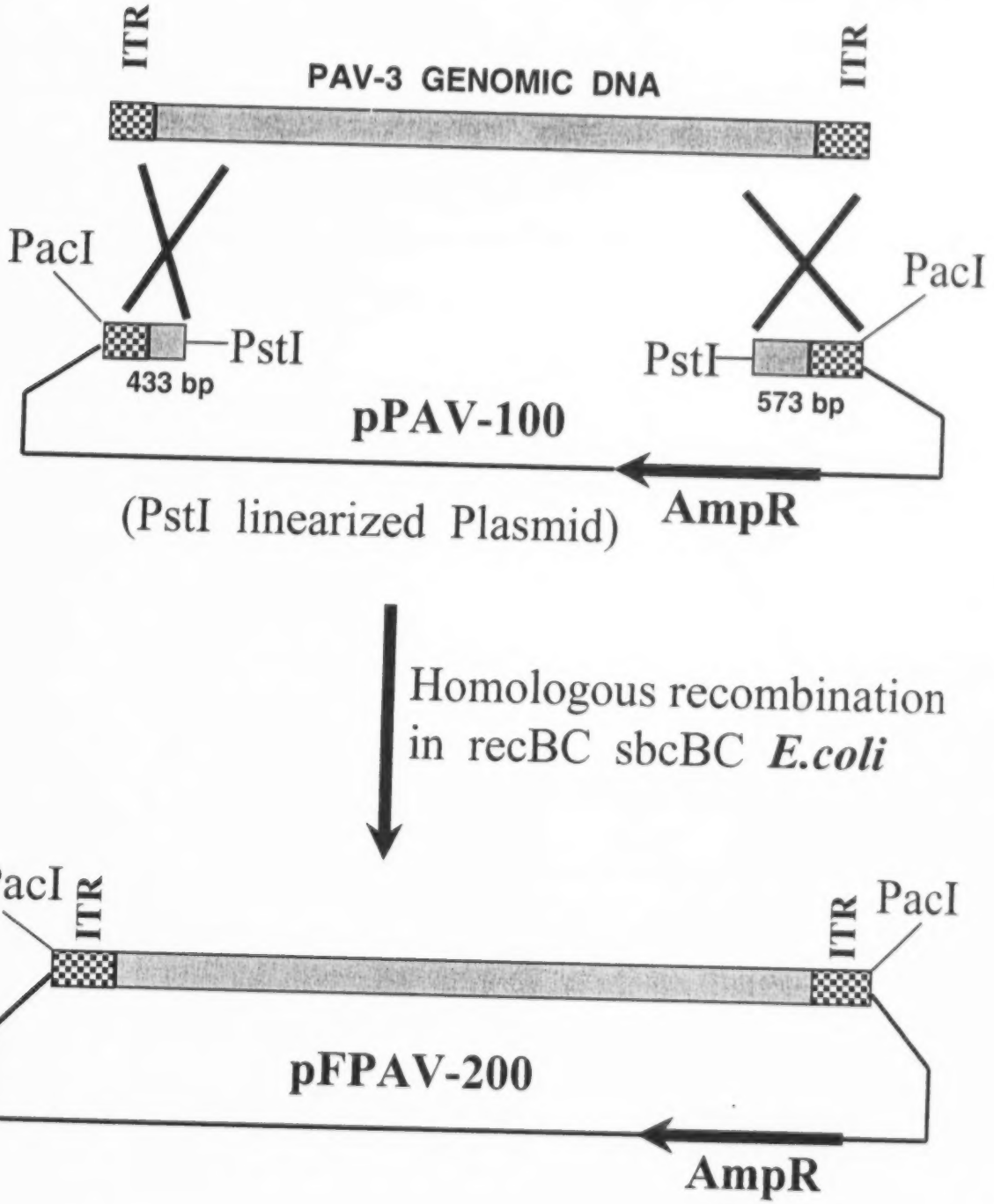
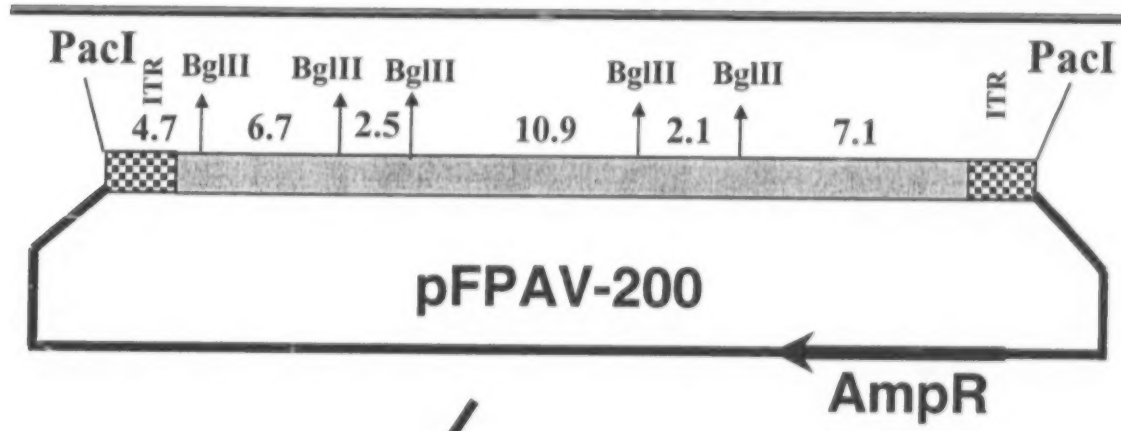


Figure 2a

PAV-3 FULL LENGTH GENOMIC CLONE IS INFECTIOUS

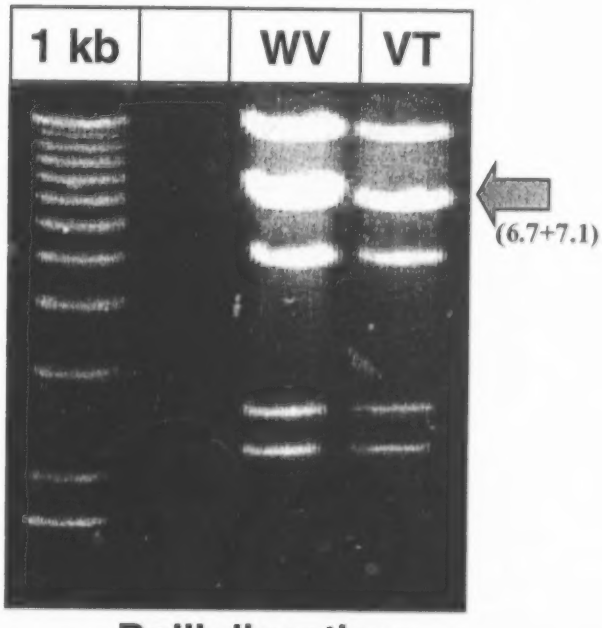


PacI digestion

Transfection of porcine cells

RE Analysis of Virion DNA

CPE appears in 12-15 days



BglII digestion

M → Mol wt marker
WV → Wild type PAV3
VT → PAV3 obtained after DNA transfection

Figure 2b

CONSTRUCTION OF E3 DELETED PAV-3 GENOMIC CLONE

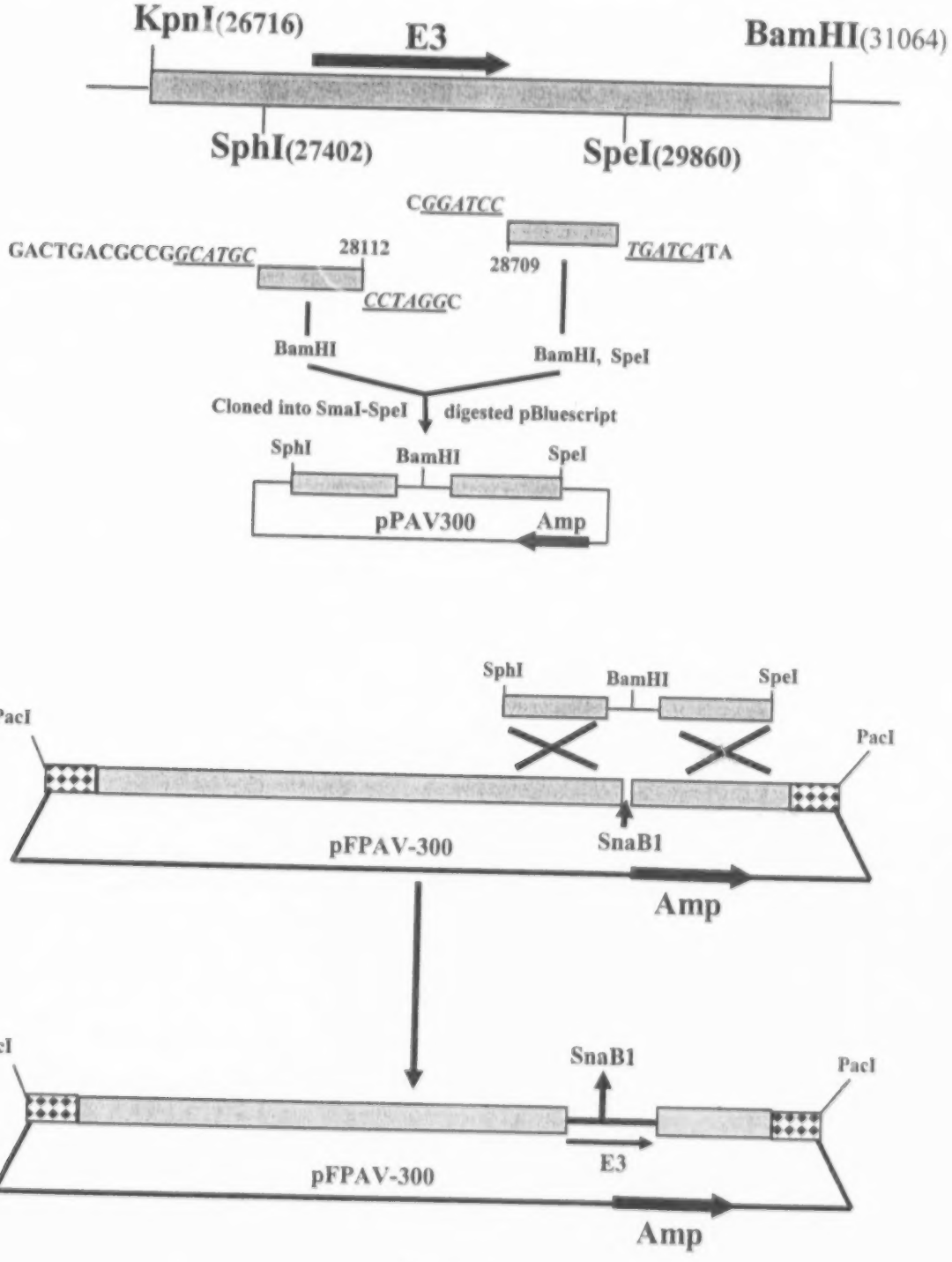
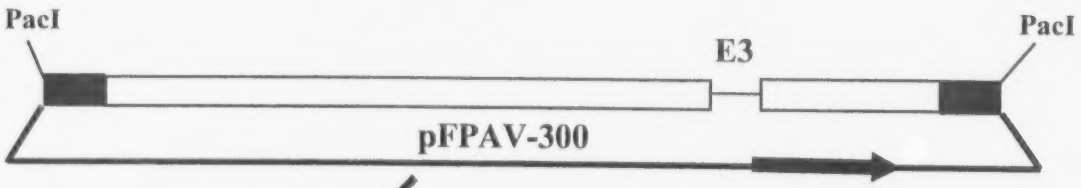


Figure 3

CONSTRUCTION OF E3 DELETED PAV-3



PacI cut

Transfect ST cells

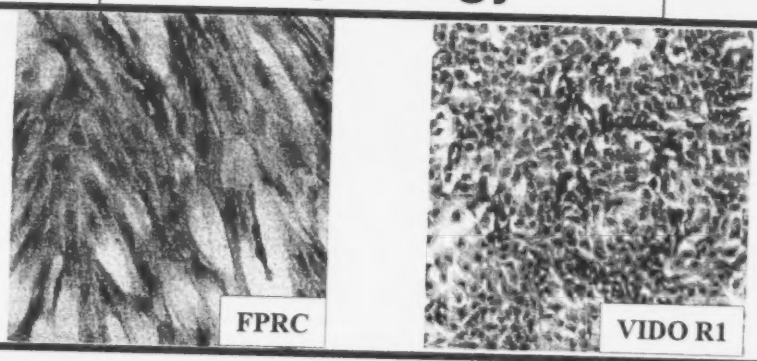
Cytopathic effects in 7 -10 days

SalI digestion

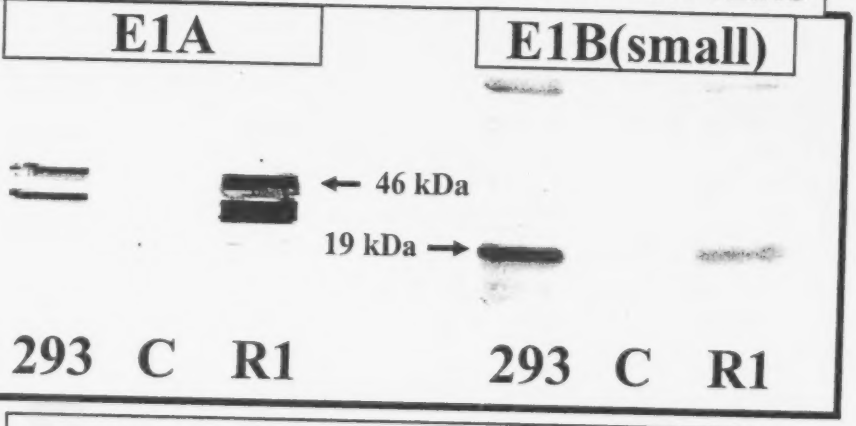
Figure 4

CHARACTERIZATION OF VIDO R1 CELL LINE

Morphology



WESTERN BLOT ANALYSIS OF E1 PROTEINS



REPLICATION OF E1A DELETED HAV-5

Cells	Virus titer
FPRC	less than 10^1 pfu/ml
VIDO R1	10^5 pfu/ml

Figure 5

CONSTRUCTION OF E1A-E3 DELETED PAV-3 GENOMIC CLONE

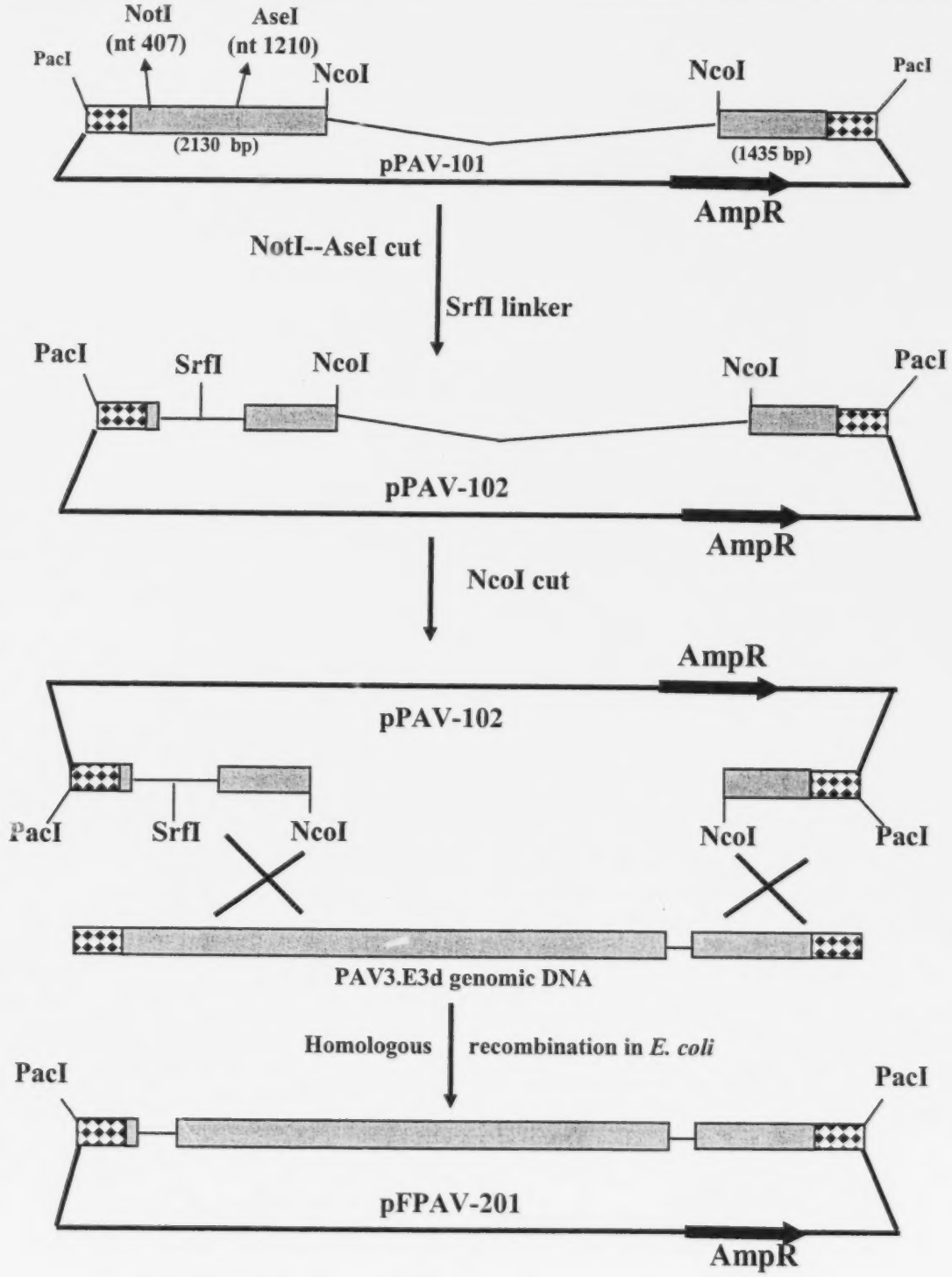


Figure 6

CONSTRUCTION OF E1A-E3 DELETED RECOMBINANT PAV-3

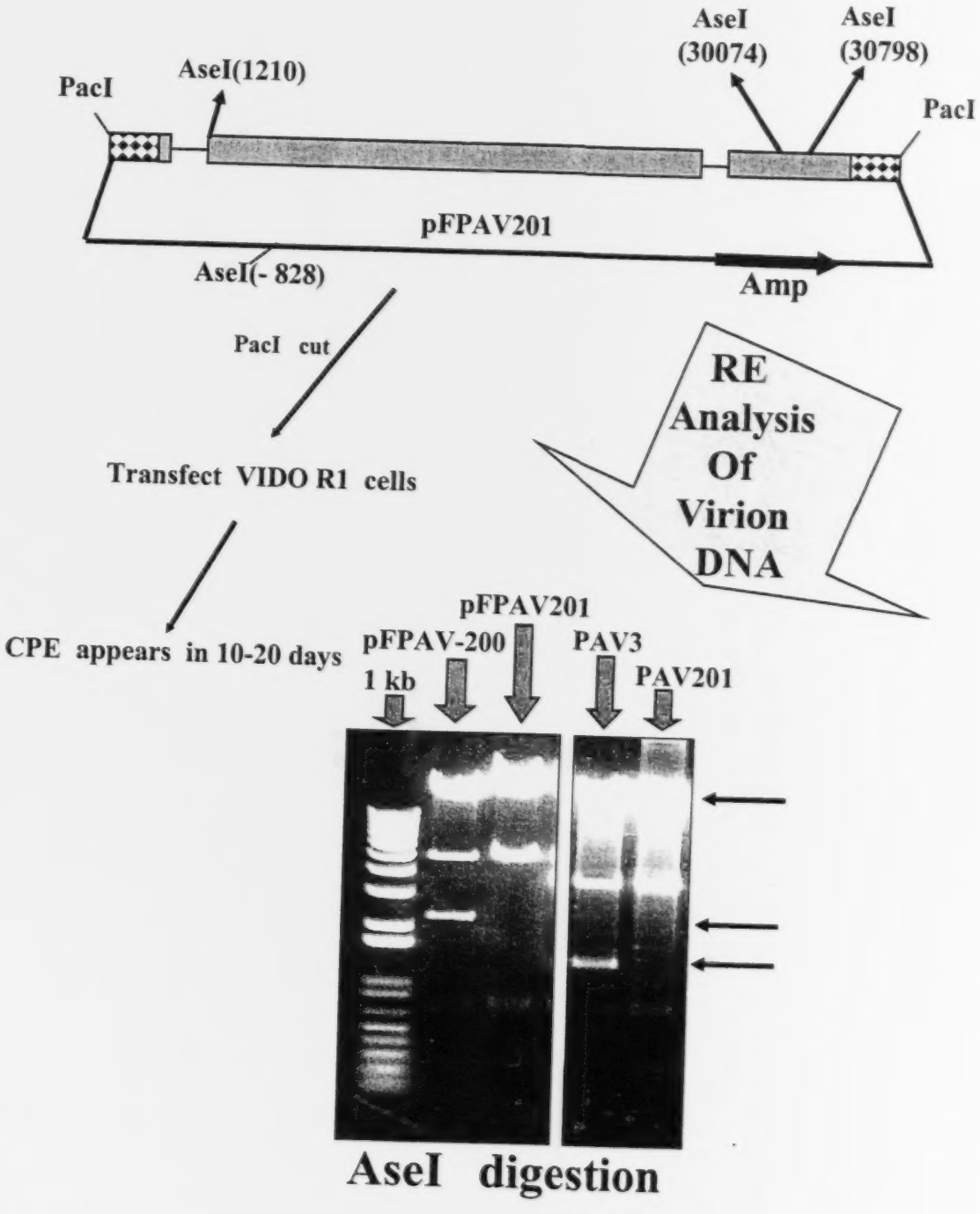


Figure 7

Construction of full length plasmid pFPAV212 (E1Bsmall+E3 deleted)

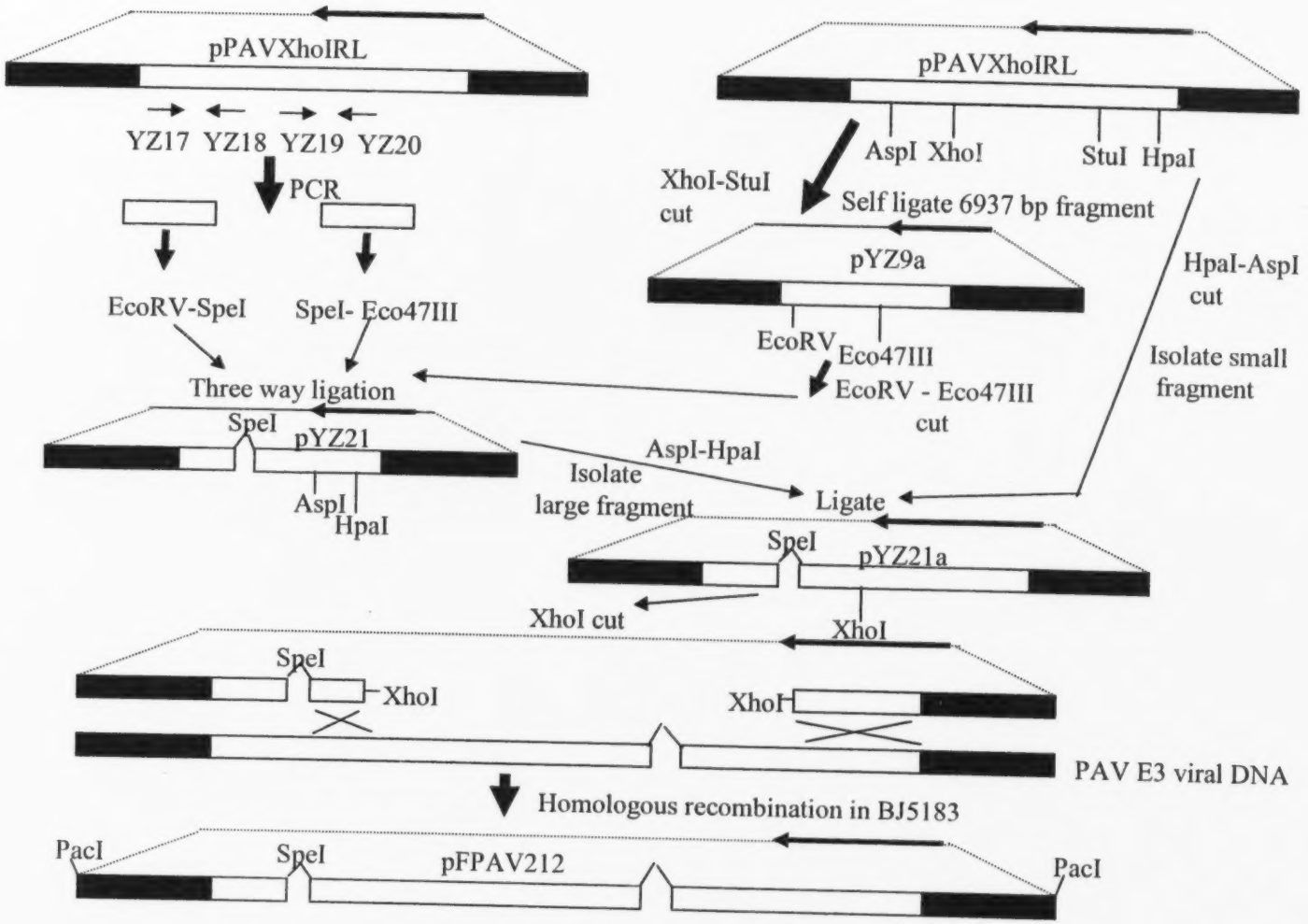
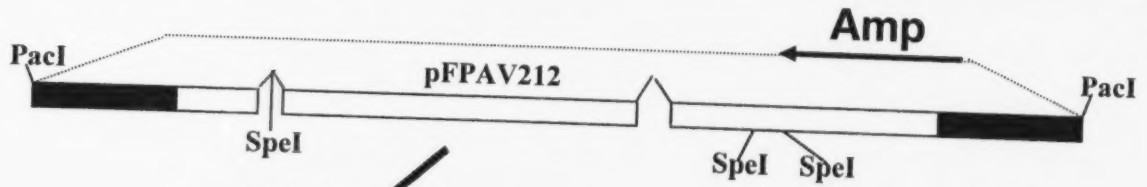


Figure 8a

Construction of PAV212

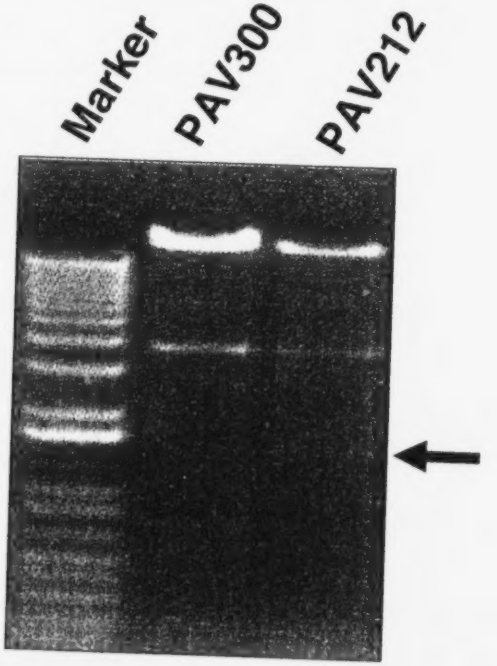


PacI digestion

Transfect porcine cells

CPE in 7 to 10 days

RE Analysis



SpeI digested

Figure 8b

Construction of full length plasmid pFPAV507 (E1Blarge insertion+E3 deletion)

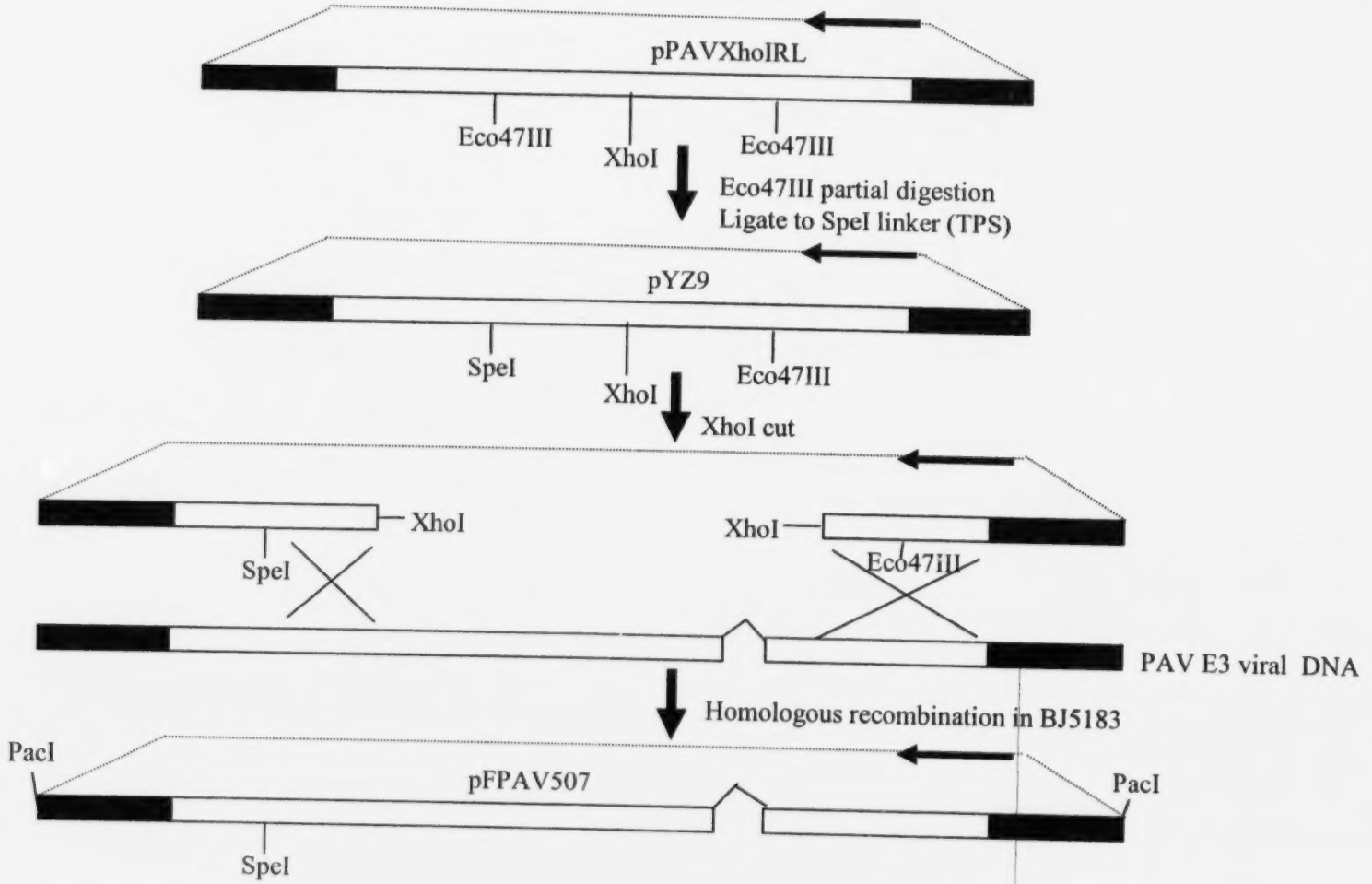


Figure 9

Construction of full length plasmid pFPAV214 (E1A+E1Bsmall+E3 deleted)

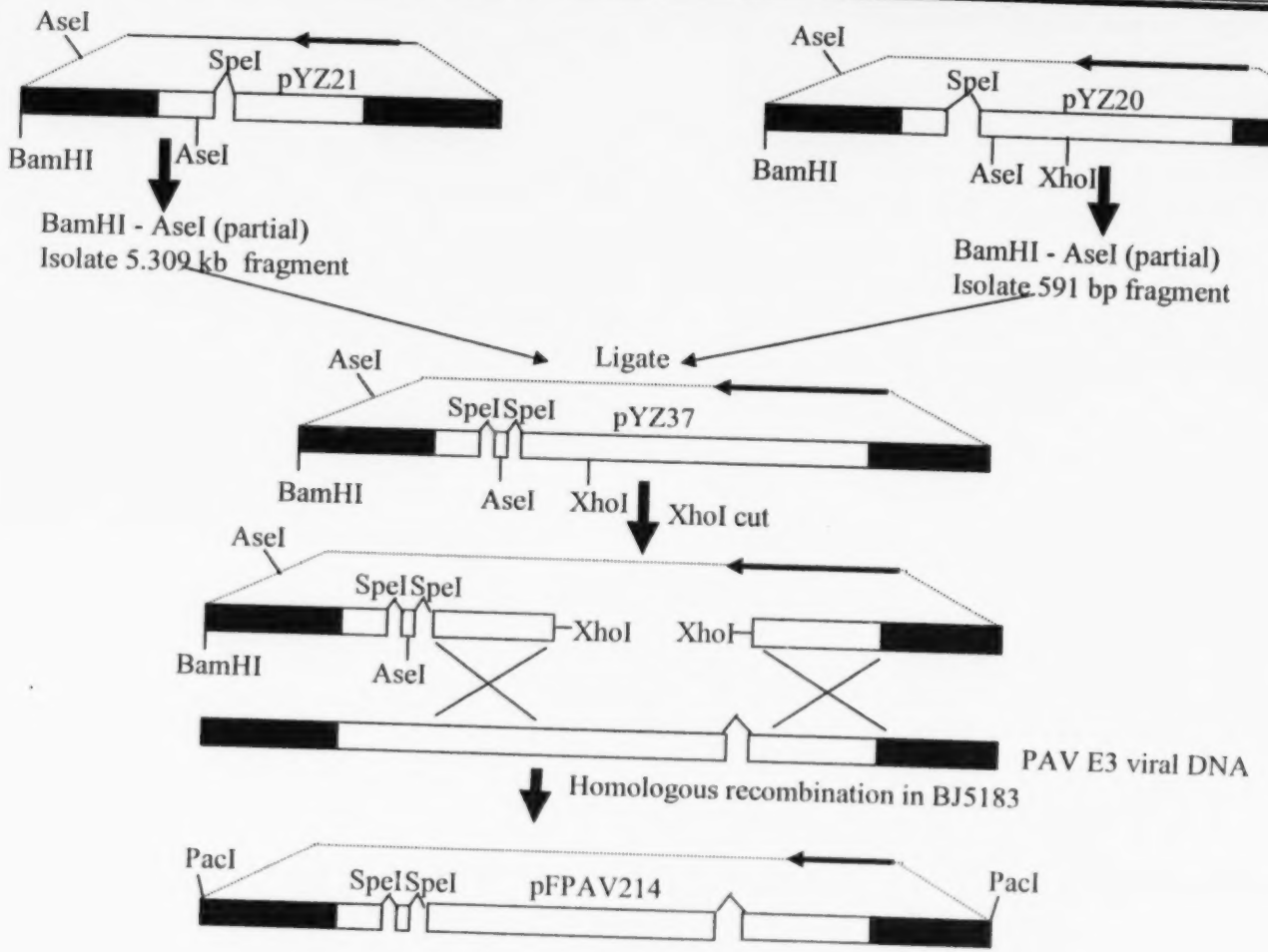


Figure 10a

Construction of PAV214

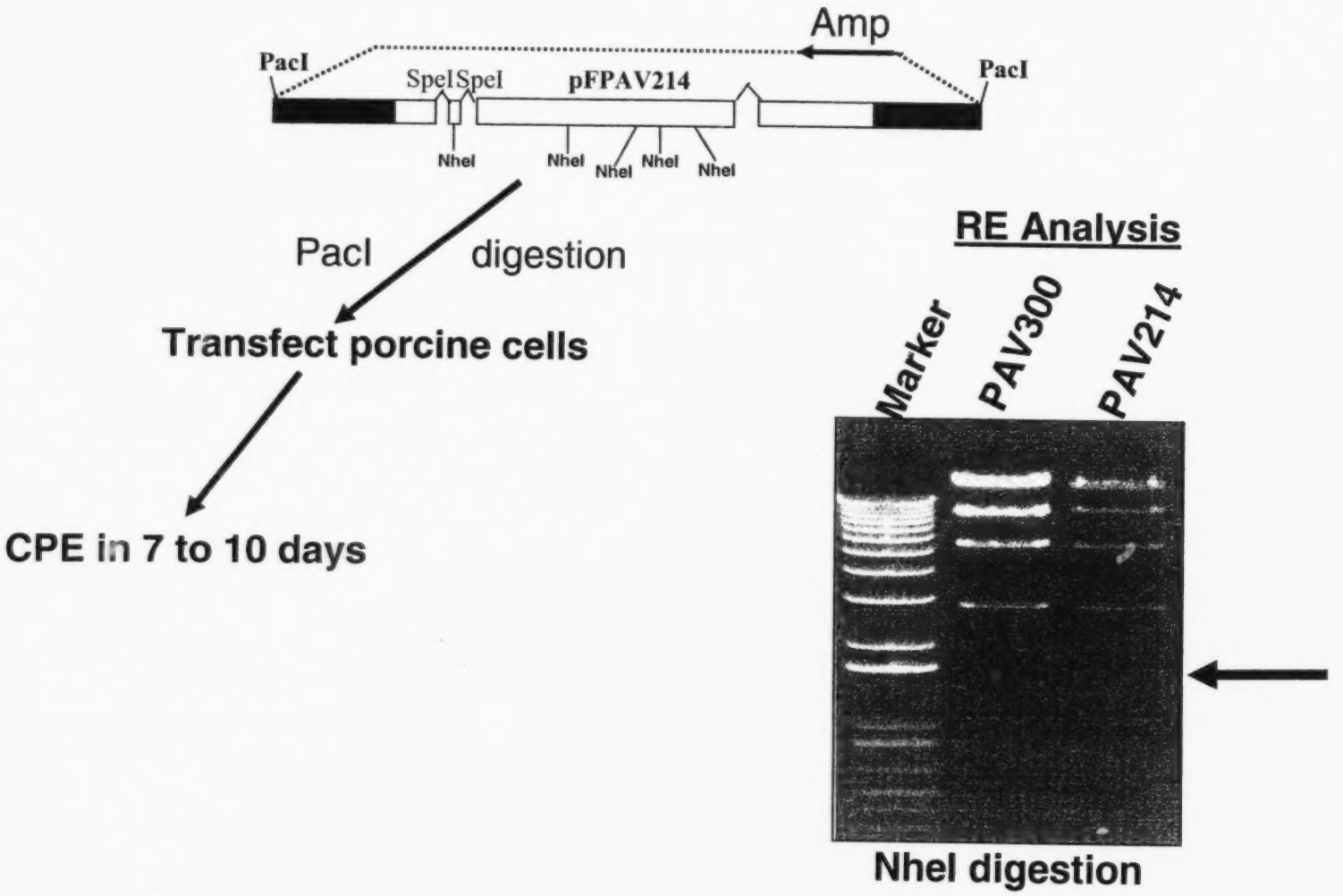
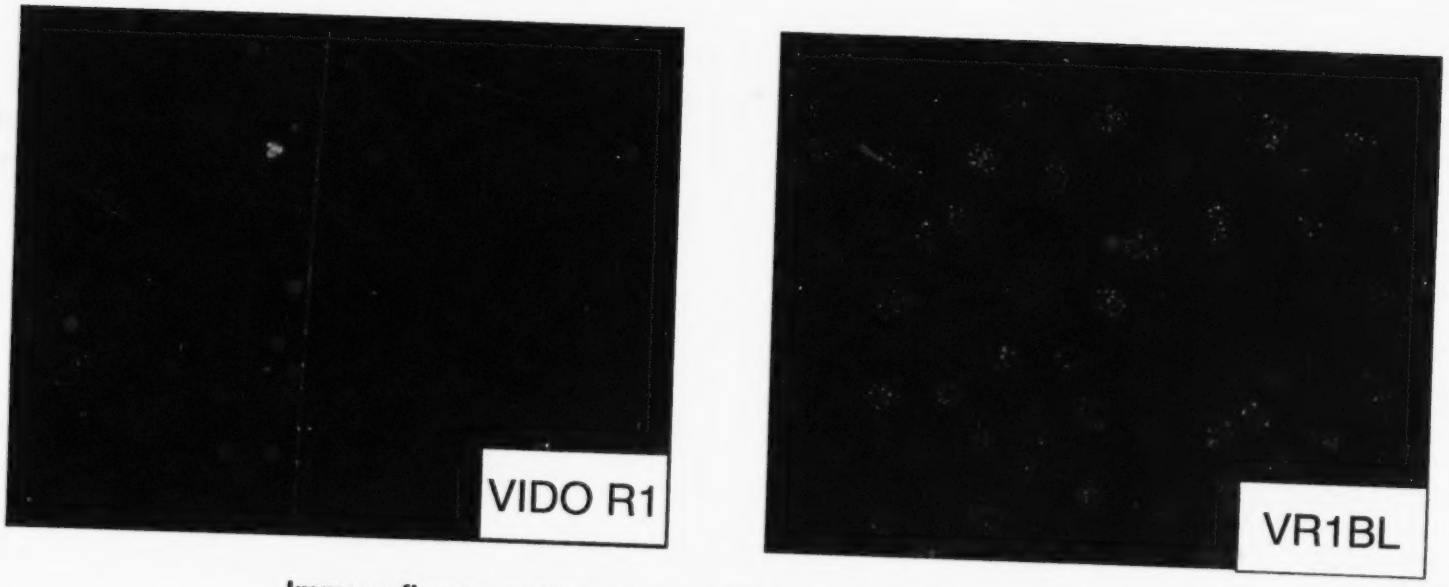


Figure 10b

Characterization of VR1BL cell line



Immunofluorescence assay using anti PAV-3 E1Blarge antibodies

Figure 11

Construction of PAV507

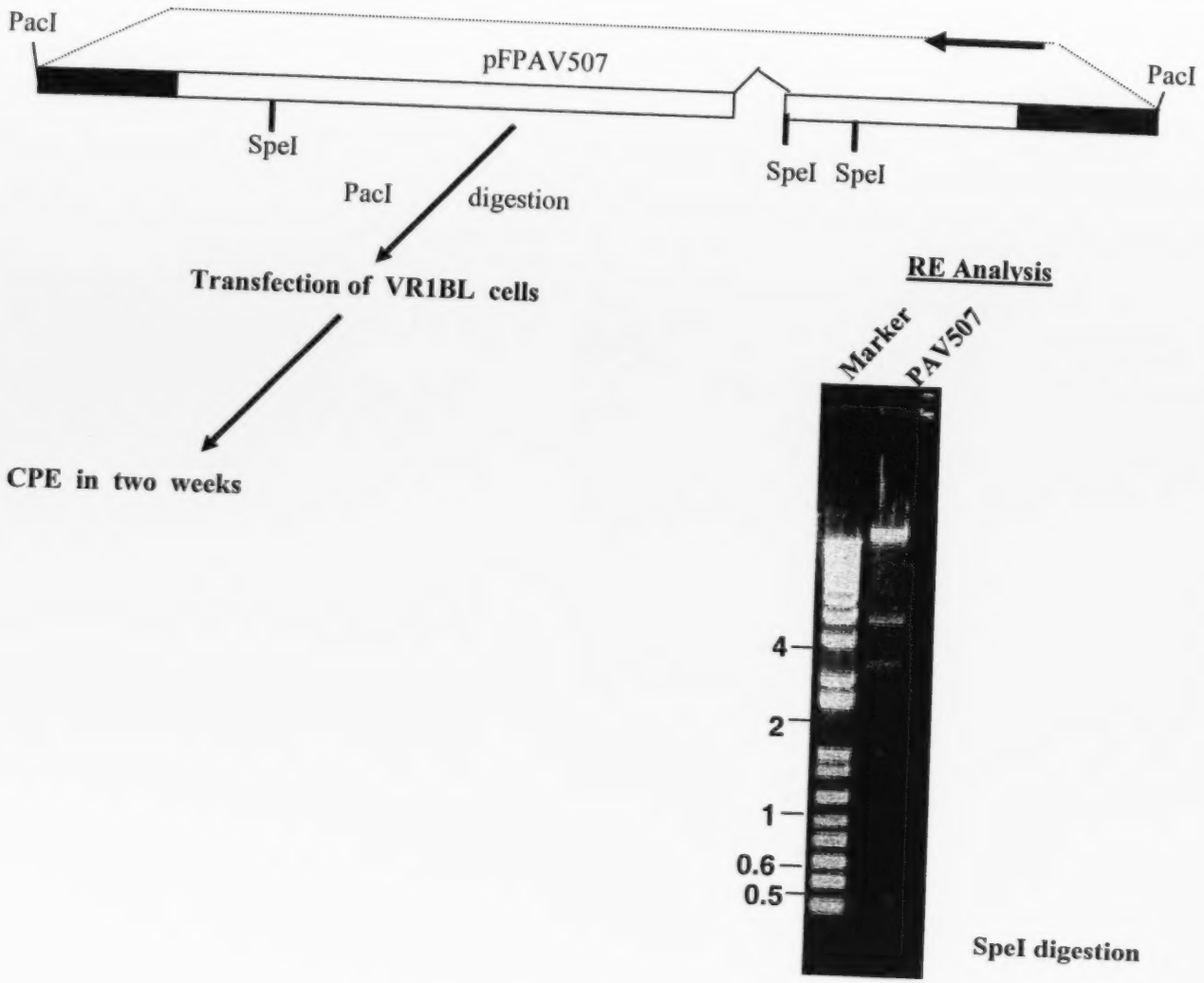


Figure 12

Construction of full length pFPAV227 (E1A+E1Bsmall+E1Blarge+ E3 deletion)

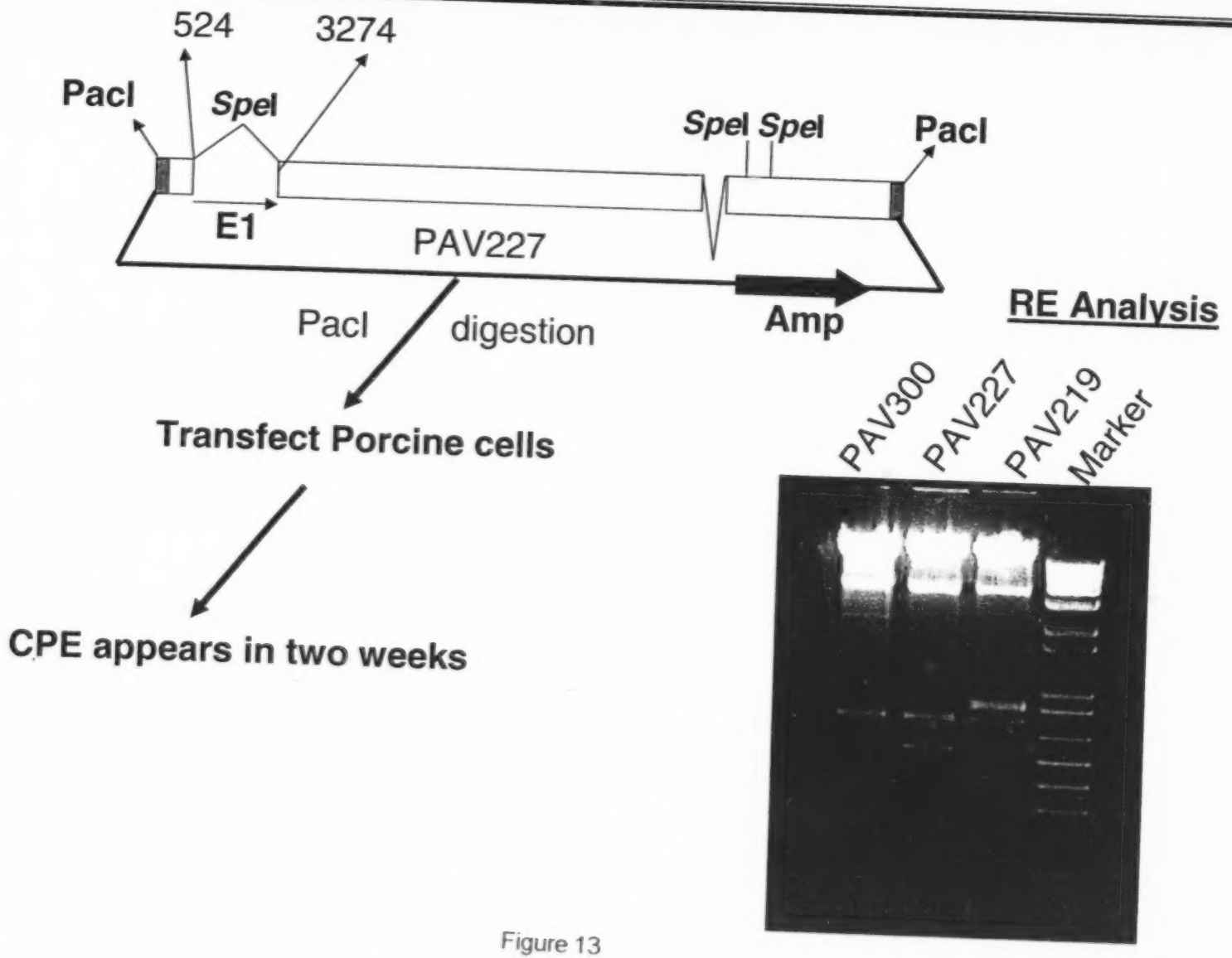


Figure 13

CONSTRUCTION OF PAV-3 GENOMIC CLONE CONTAINING E4 INSERTION

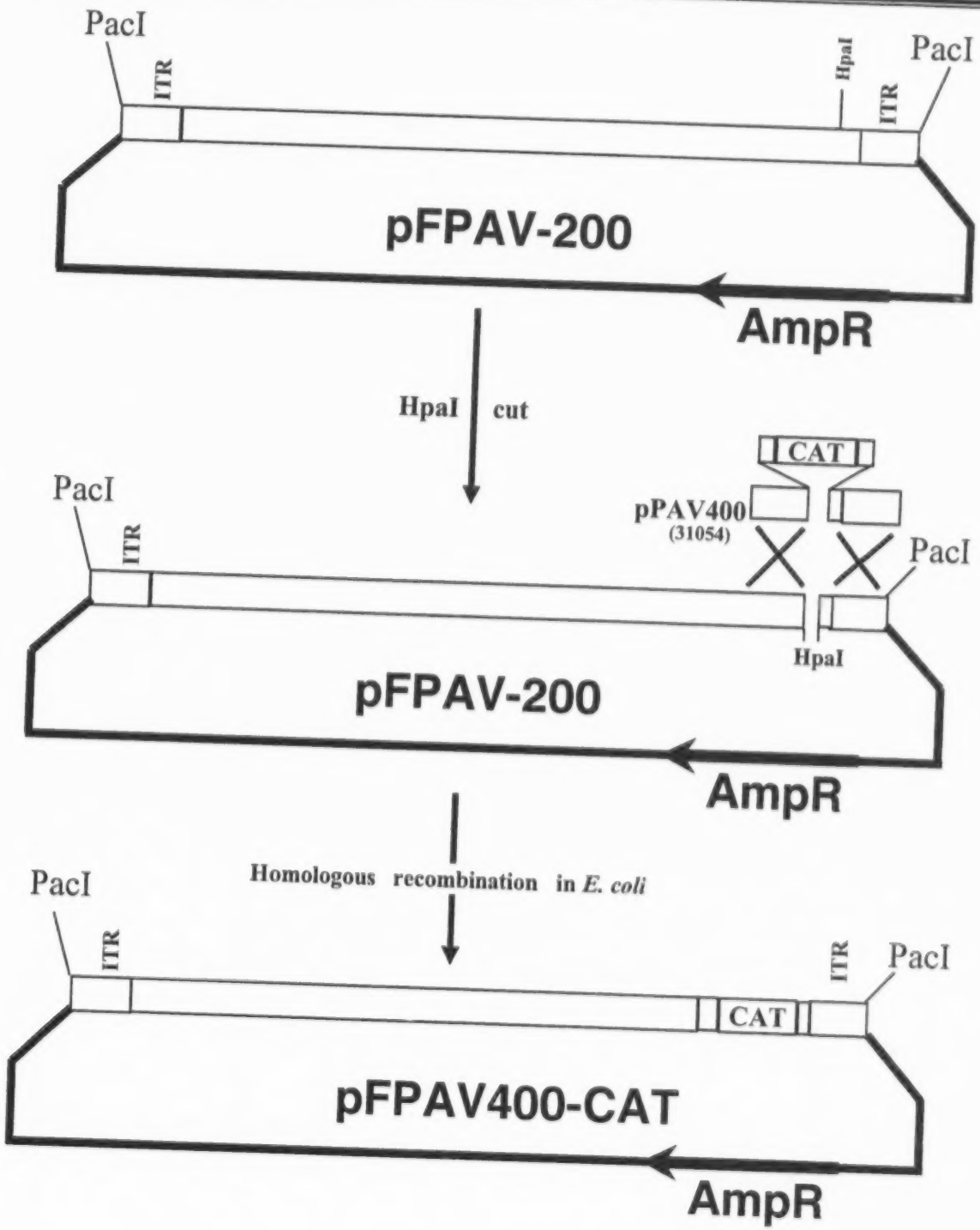


Figure 14a

Construction of PAV400 (CAT in E4)

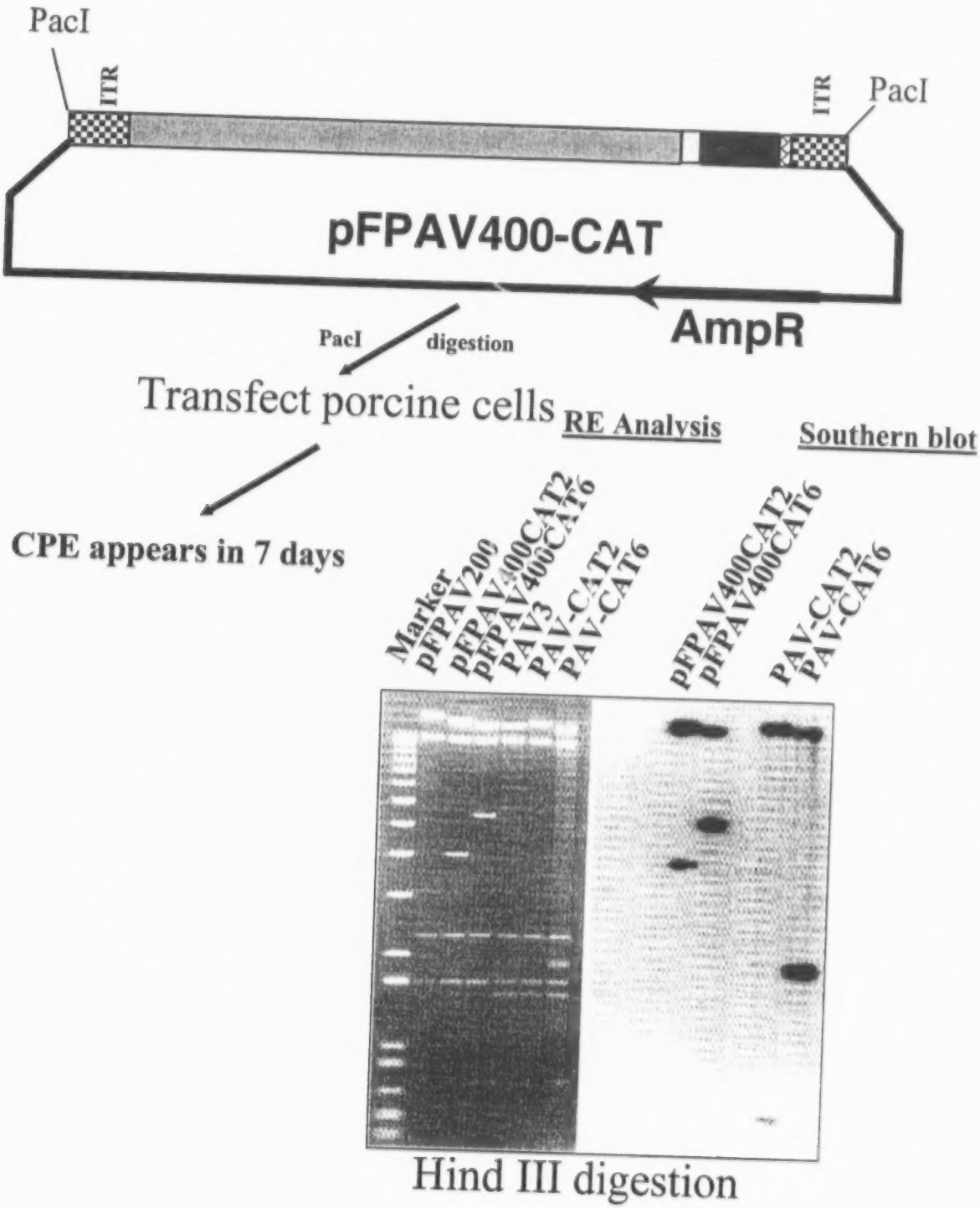


Figure 14b

Construction of full-length plasmids with E4 deletions

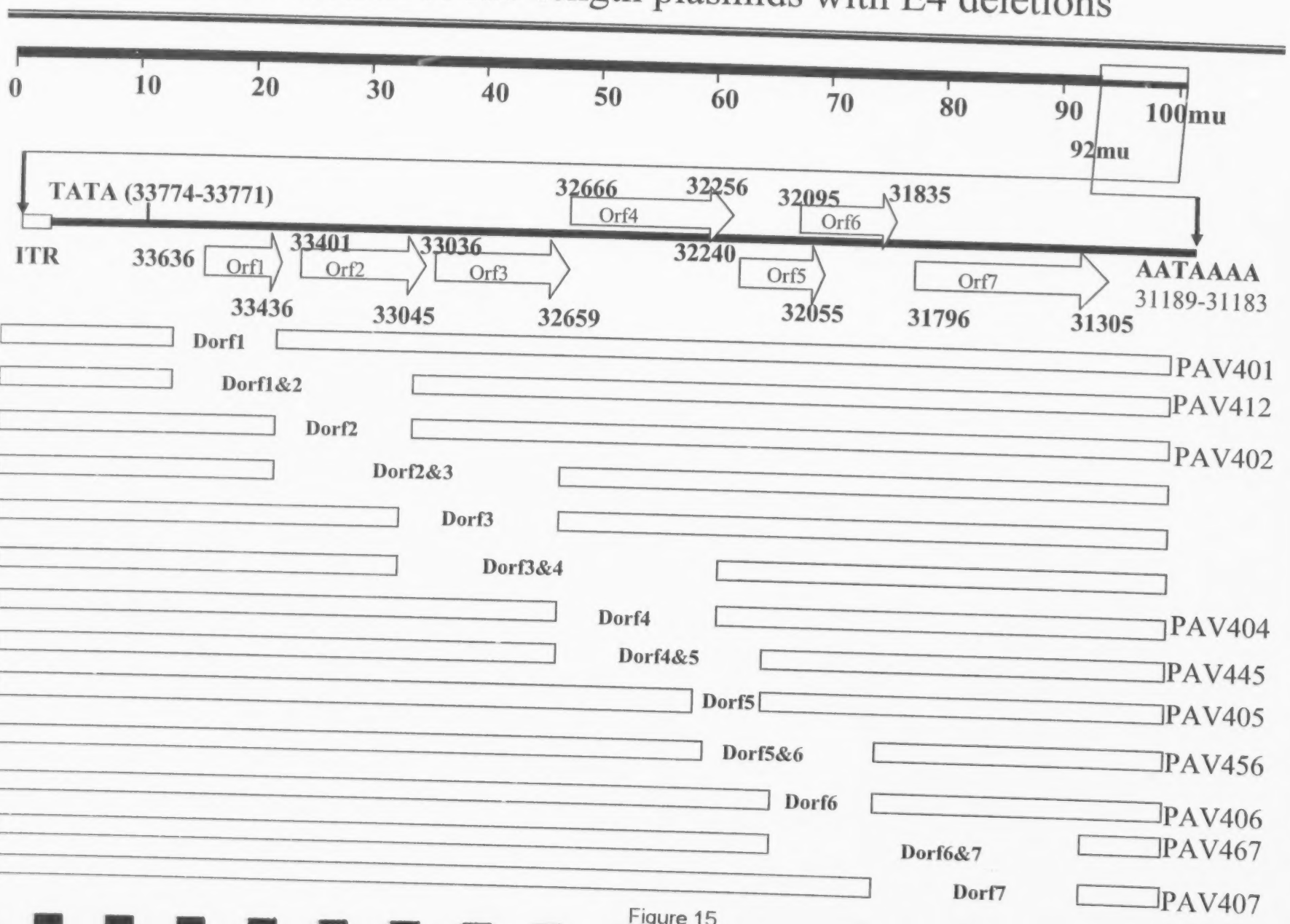
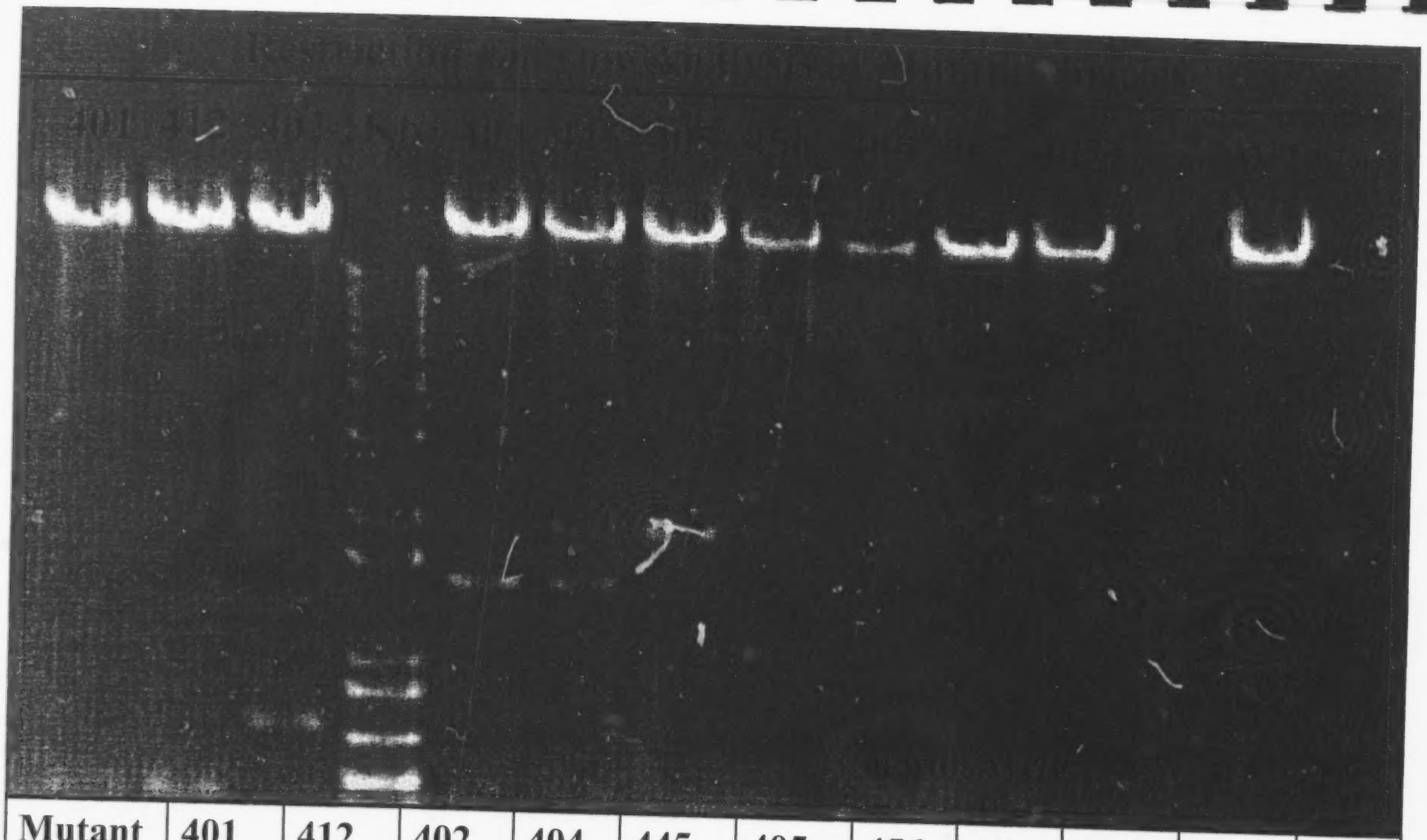
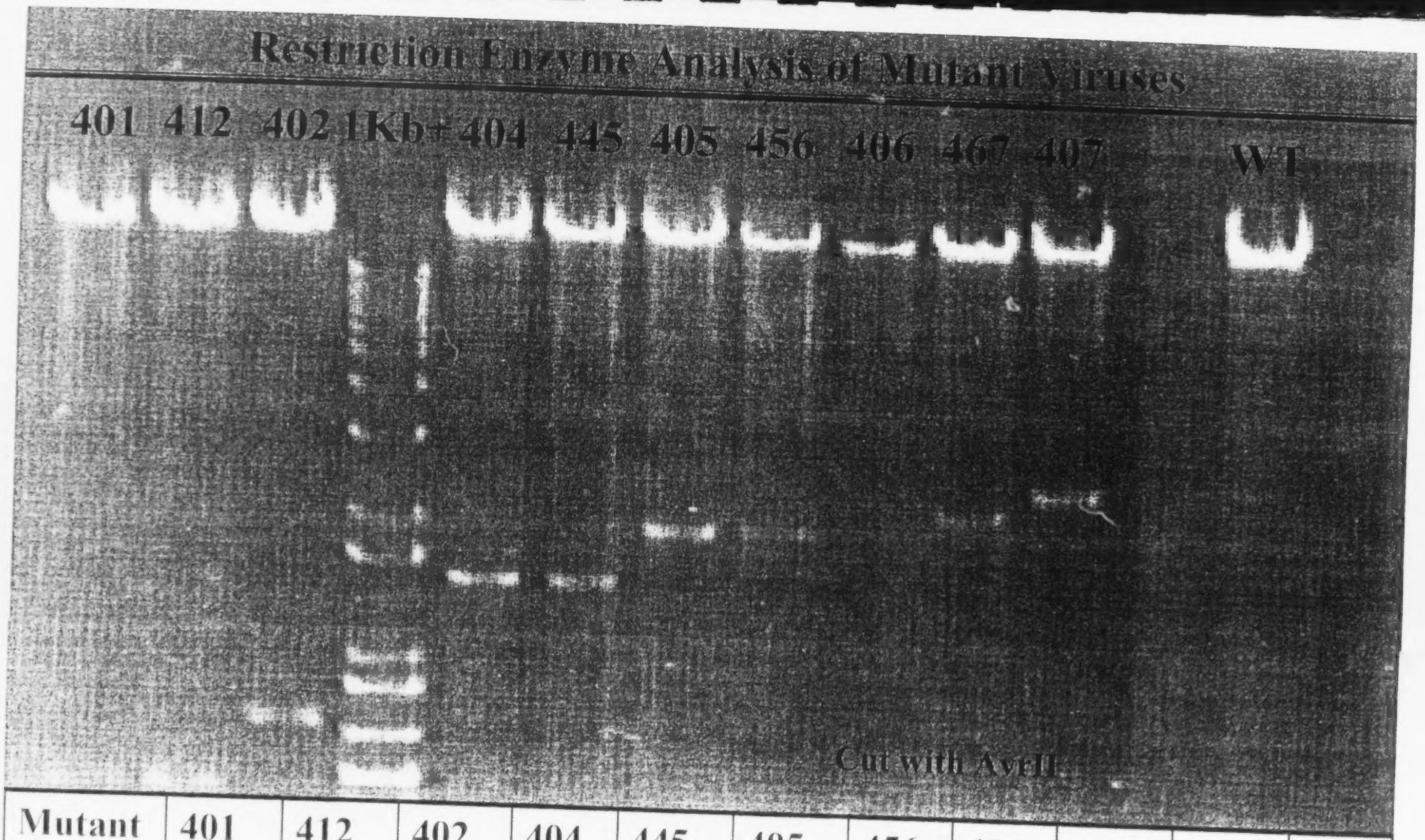


Figure 15



Mutant Viruses	401	412	402	404	445	405	456	406	467	407	WT
Expected	466	466	699	1436	1436	1855	1855	2049	2049	2289	
Sizes	33436	33044	33044	32264	32103	32103	31834	31834	31303	31303	34097

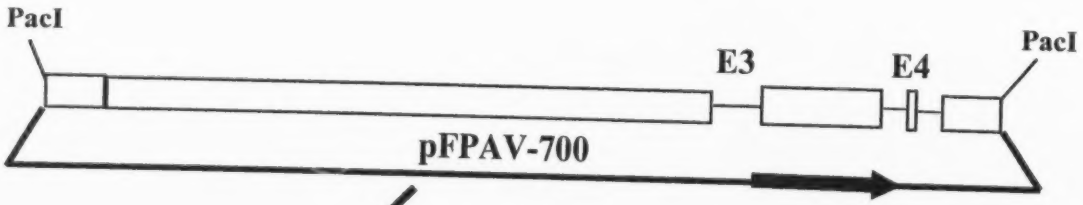
Figure 16



Mutant Viruses	401	412	402	404	445	405	456	406	467	407	WT
Expected	466	466	699	1436	1436	1855	1855	2049	2049	2289	
Sizes	33436	33044	33044	32264	32103	32103	31834	31834	31303	31303	34097

Figure 16

CONSTRUCTION OF RECOMBINANT PAV700



PacI cut

Transfect ST cells

Cytopathic effects in 7 -10 days

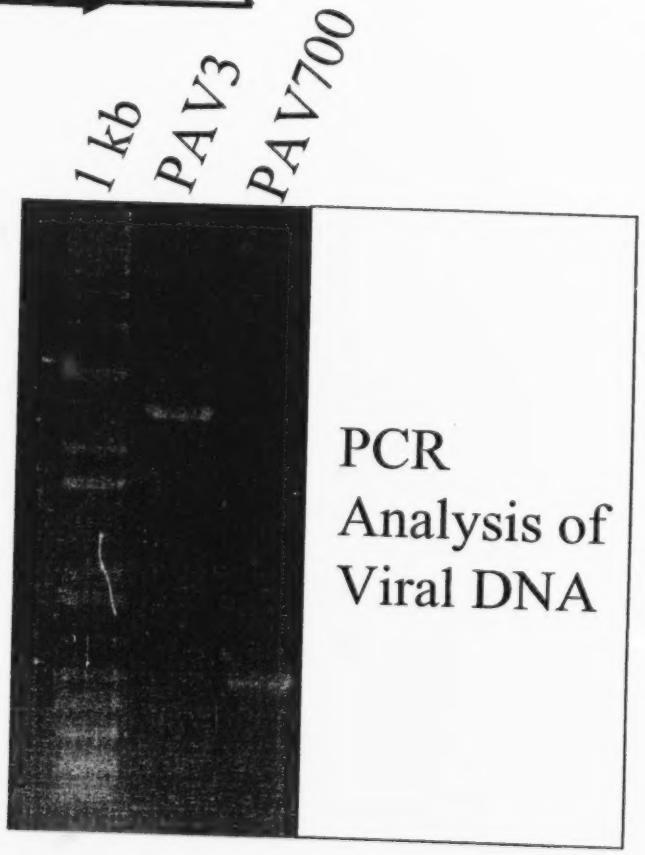
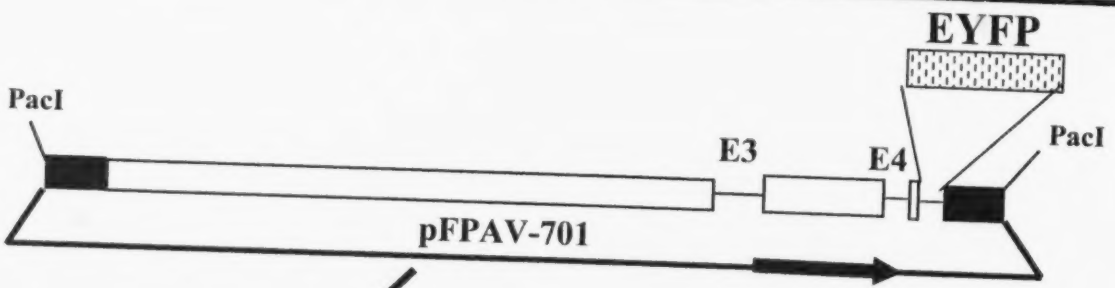


Figure 17

CONSTRUCTION OF RECOMBINANT PAV701



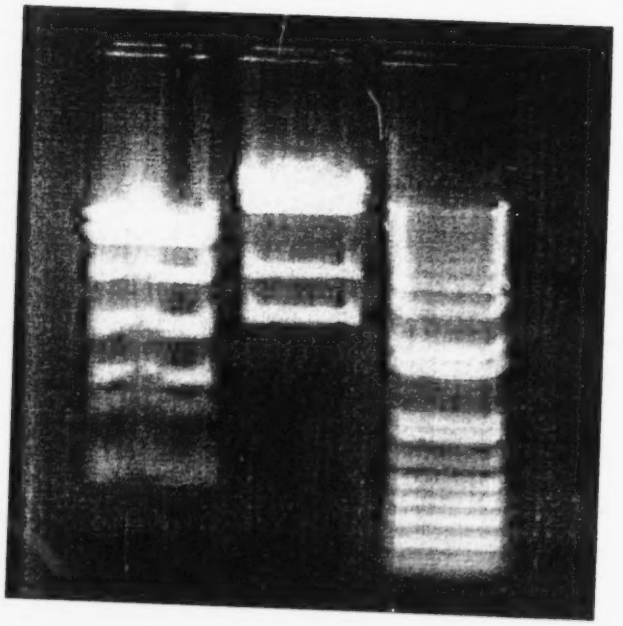
PacI cut

Transfect ST cells

Cytopathic effects in 10 -12 days

RE analysis

AatII BamHI



EYFP Expression in Infected cells

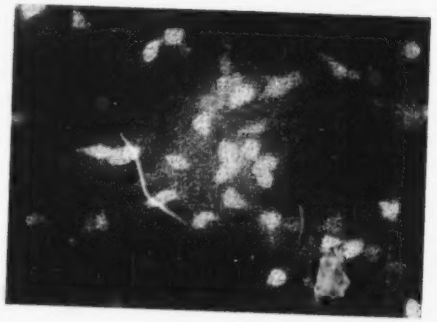
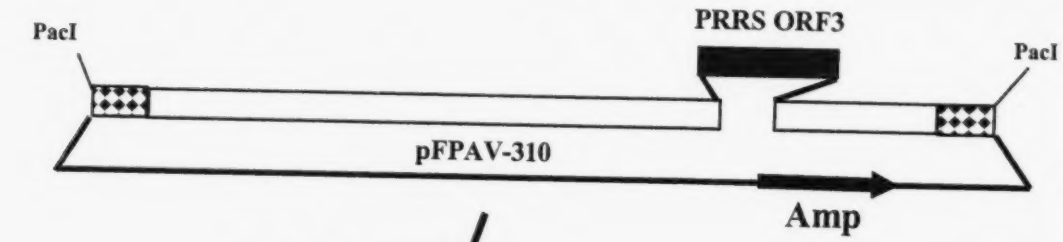


Figure 18

CONSTRUCTION OF E3 DELETED PAV-3 CONTAINING PRRS ORF3 (PAV310)

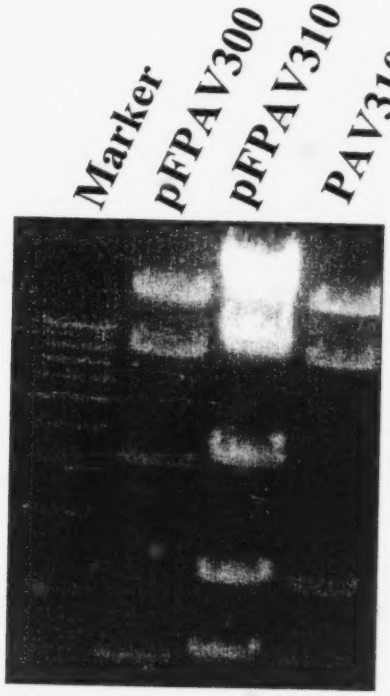


PacI digestion

Transfection of Porcine cells

CPE appears two weeks

RE Analysis of Virion DNA



BamHI digestion

Figure 19

CONSTRUCTION OF E3 DELETED PAV-3 CONTAINING PRRS ORF5 (PAV312)

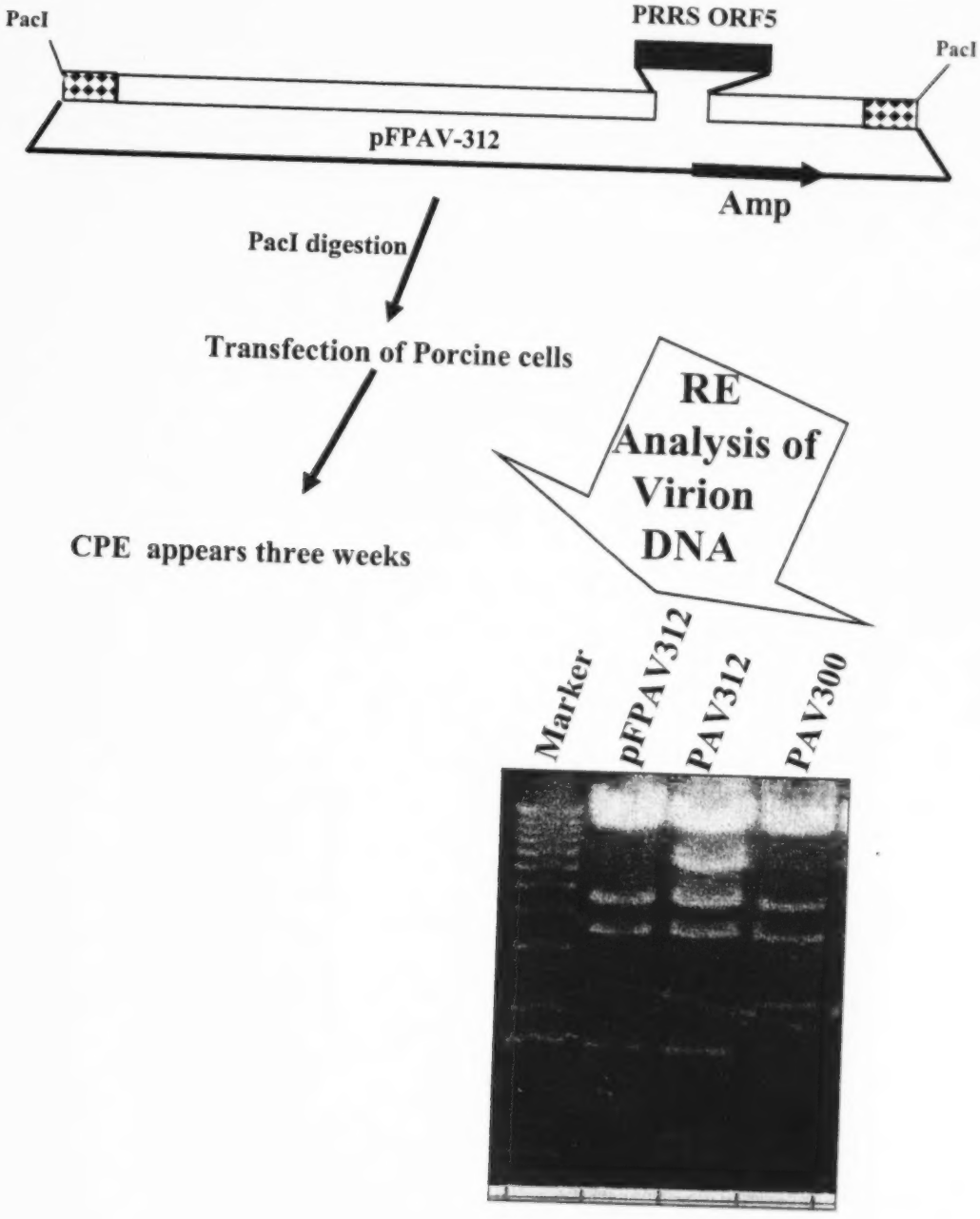


Figure 20

PRRS IgG ELISA Titers

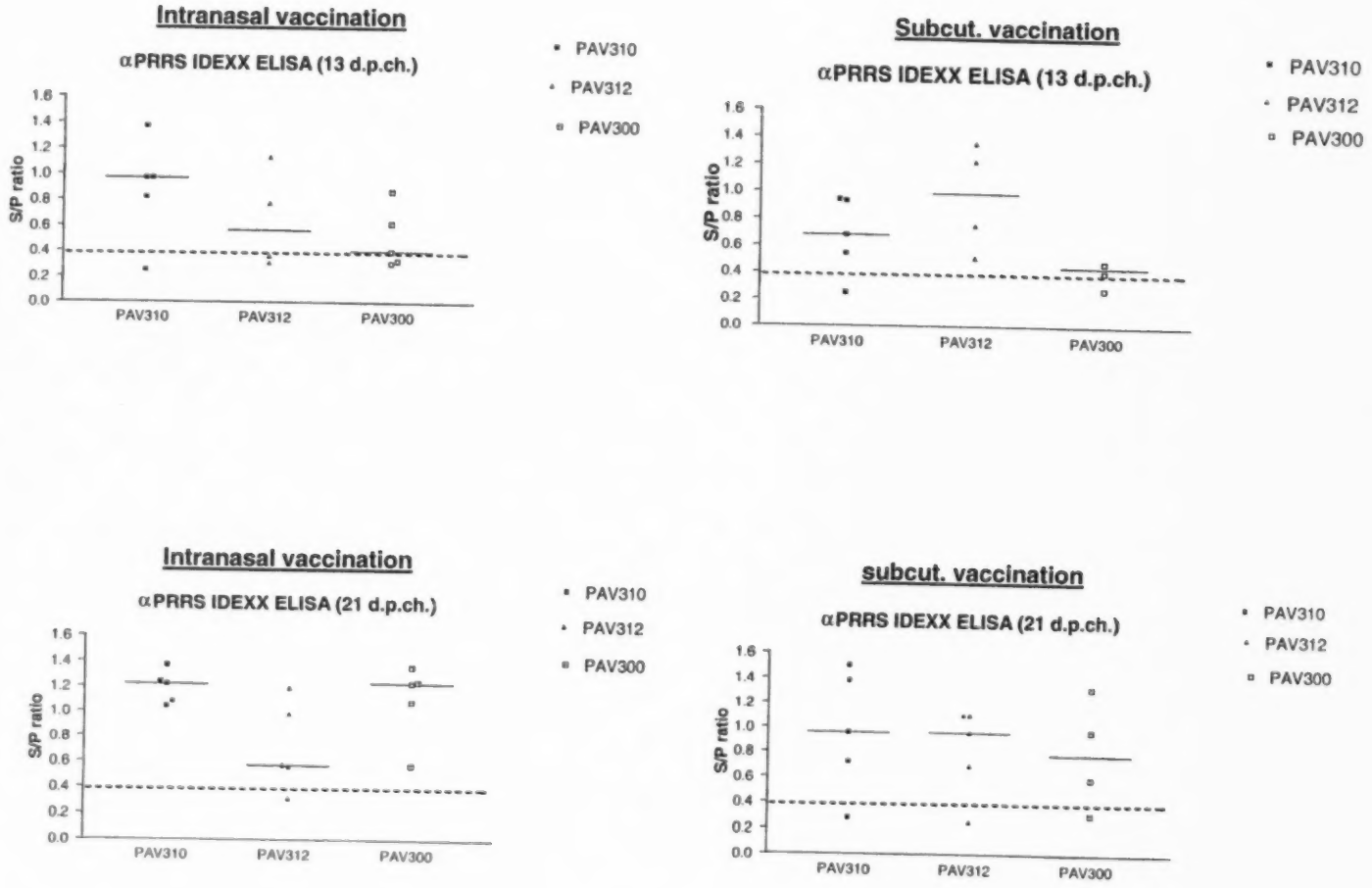
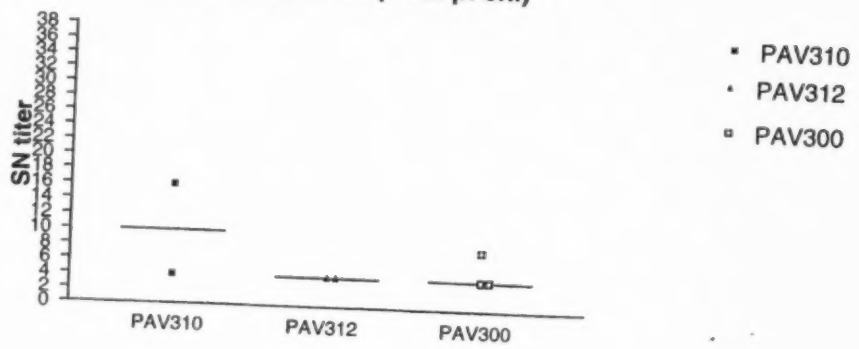


Figure 21

Serum PRRS neutralization titers

Intranasal vaccination



Subcut. vaccimation

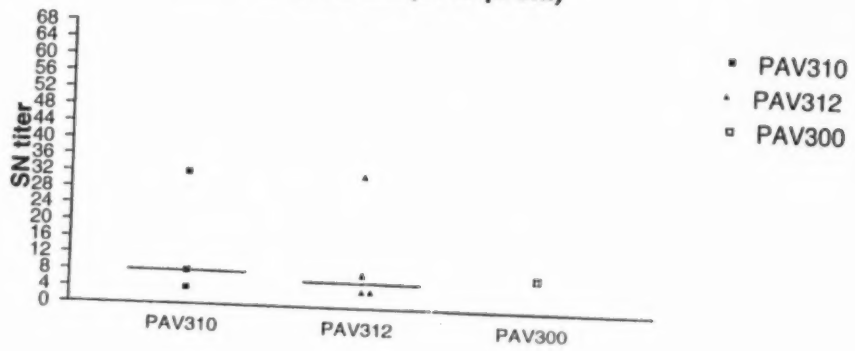


Figure 22

Rectal Temperatures of pigs

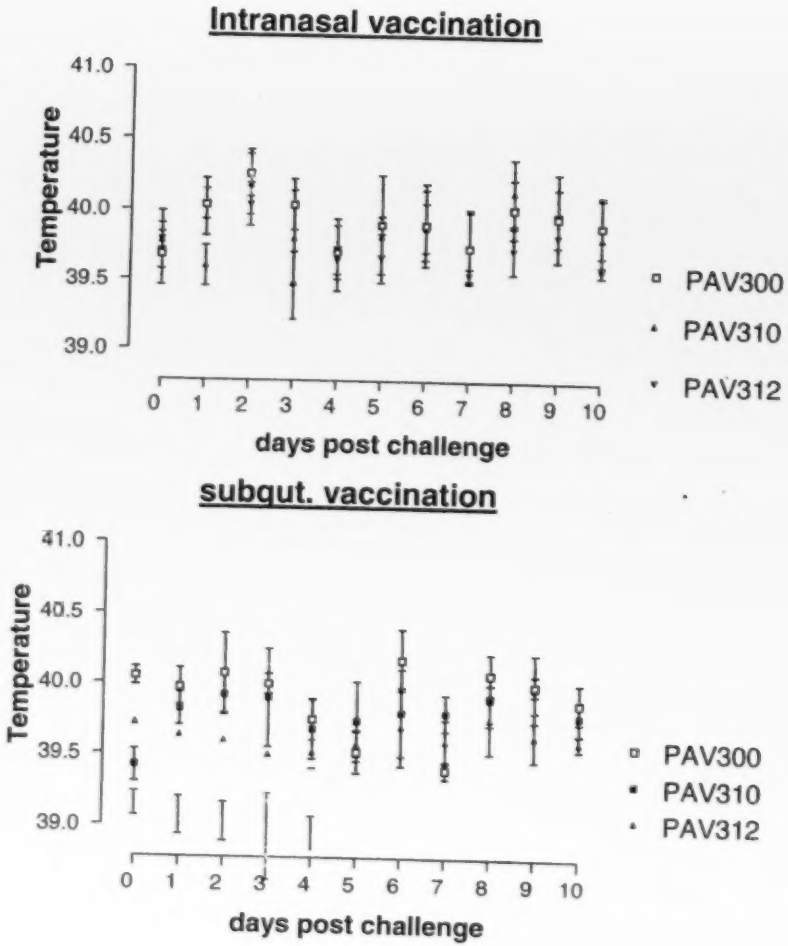


Figure 23